FACULTAD DE CIENCIAS EXPERIMENTALES

Departamento de Biología Molecular e Ingeniería Bioquímica

TESIS DOCTORAL

La industria de los aceites de oliva y el tratamiento de sus aguas residuales mediante bioprocesos combinados basados en operaciones fisicoquímicas y cultivos de microalgas

Ana Malvis Romero

Sevilla, 2020

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Memoria presentada para optar al Título de Doctor por la Universidad Pablo de Olavide bajo la dirección de los profesores Dr. Gassan Hodaifa Meri y Dr. Sebastián Sánchez Villasclaras

Aspirante al Título de Doctor

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Sevilla, 2020

GASSAN HODAIFA MERI, PROFESOR TITULAR EN LA UNIVERSIDAD PABLO DE OLAVIDE EN EL DEPARTAMENTO DE BIOLOGÍA MOLECULAR E INGENIERÍA BIOQUÍMICA Y SEBASTIÁN SÁNCHEZ VILLASCLARAS, PROFESOR CATEDRÁTICO EN LA UNIVERSIDAD DE JAÉN EN EL DEPARTAMENTO DE INGENIERÍA QUÍMICA, AMBIENTAL Y DE LOS MATERIALES,

CERTIFICAN: Que el presente trabajo titulado "La industria de los aceites de oliva y el tratamiento de sus aguas residuales mediante bioprocesos combinados basados en operaciones fisicoquímicas y cultivos de microalgas" ha sido realizado en los laboratorios del Área de Ingeniería Química del Departamento de Biología Molecular e Ingeniería Bioquímica, en la Facultad de Ciencias Experimentales, bajo la dirección de los Profesores Dr. Gassan Hodaifa Meri y Dr. Sebastián Sánchez Villasclaras, por Dª Ana Malvis Romero para optar al Título de Doctor por la Universidad Pablo de Olavide, dentro del Programa de Doctorado "Biotecnología, Ingeniería y Tecnología Química".

Sevilla, Julio 2020

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RESUMEN

Esta Tesis Doctoral se ha llevado a cabo en los laboratorios del Área de Ingeniería Química del Departamento de Biología Molecular e Ingeniería Bioquímica, de la Universidad Pablo de Olavide de Sevilla. Este trabajo de investigación ha estudiado la biorremediación de aguas residuales (urbanas e industriales) mediante la combinación de tratamientos fisicoquímicos y cultivos de microalgas. Además, propone nuevos métodos para determinar la estabilidad de los aceites de oliva.

España atesora el mayor olivar del mundo, además, es líder mundial en producción, comercialización y exportación de aceites de oliva, lo que se traduce en un enorme valor económico, social, medioambiental y cultural. La vital importancia de la industria oleícola se muestra en que la producción española representa el 60% de la producción total de la Unión Europea y el 50% de la mundial. Sin embargo, esta gran producción de aceites de oliva ha provocado que la industria oleícola se enfrente actualmente a dos importantes desafíos respecto a la gestión de los residuos generados y a la autentificación y trazabilidad de los aceites de oliva.

Por un lado, la producción de aceites de oliva genera grandes volúmenes de aguas residuales en las almazaras (ARAs). La composición fisicoquímica de las ARAs se caracteriza por una elevada heterogeneidad y su composición depende del proceso empleado para la extracción del aceite de oliva. Dicho proceso se puede llevar a cabo mediante proceso discontinuo (prensa) o continuo (por centrifugación). A su vez, este último se puede realizar empleando un 'Decánter' con dos o tres salidas, siendo el proceso de centrifugación con un 'Decánter' de dos salidas (una para el aceite de oliva y otra el alperujo) el utilizado en España. Las ARAs generadas mediante este sistema se caracterizan por poseer un pH ligeramente ácido y una elevada carga orgánica que además de incluir polisacáridos, azúcares, ácidos orgánicos, etc. incluye una alta concentración de compuestos fenólicos, principales responsables de la toxicidad de las ARAs debido a su gran fitotoxicidad y actividad antimicrobiana. Actualmente, la acumulación de las ARAs en grandes balsas con poca profundidad para la evaporación del agua en los meses de verano representa el sistema de gestión más empleado por las almazaras. Sin embargo, este sistema conlleva numerosos inconvenientes tales como la contaminación de aguas subterráneas, la generación de malos olores o la proliferación de insectos. Por todo ello, el tratamiento de estas aguas residuales representa un gran desafío para la industria oleícola.

En este trabajo de investigación se propone un novedoso bioproceso basado en la combinación de operaciones fisicoquímicas con el cultivo de microalgas como sistema integral de tratamiento de las ARAs. El objetivo de dicho proceso es la obtención de un agua final de alta calidad que sea apta para su reutilización en actividades industriales, riego o para su vertido directo en cauces naturales. A su vez, la biomasa microalgal resultante es rica en compuestos energéticos por lo que tiene un gran valor económico.

Para ello, se han diseñado y ejecutado tres bioprocesos y se ha estudiado la eficacia de cada uno de ellos en el tratamiento de las ARAs, así como en el crecimiento microalgal. El primero de ellos ha consistido en un pretratamiento fisicoquímico primario (floculación-sedimentación, fotólisis con luz UV artificial y microfiltración) seguido del cultivo de *Chlorella pyrenoidosa* en diferentes concentraciones de ARAs pretratadas. El segundo bioproceso consistió en un tratamiento primario (floculación-sedimentación y microfiltración) seguido del cultivo de *Scenedesmus obliquus* en diferentes concentraciones de ARAs pretratada. El tercero ha estudiado la combinación de ARAs (pretratadas mediante floculación-sedimentación y fotólisis con luz UV artificial) con aguas residuales urbanas para el tratamiento simultáneo de ambas aguas residuales y la formación de diferentes medios de cultivo para el crecimiento de *Chlorella pyrenoidosa*. En las tres series experimentales se ha estudiado la composición fisicoquímica de las ARAs a lo largo tanto del tratamiento primario como del cultivo microalgal mediante la determinación de parámetros como los compuestos fenólicos totales (CFTs), el carbono total (CT), el carbono orgánico total (COT), el carbono inorgánico (IC), el nitrógeno total (NT), etc. Además, se ha evaluado la cinética del crecimiento microalgal en base a la velocidad específica máxima de crecimiento (μ_m) y la productividad volumétrica en biomasa (P_b), se ha determinado también la composición bioquímica de la biomasa final y la calidad del agua residual tratada final obtenida.

Los resultados obtenidos demuestran que la realización de un pretratamiento primario basado en la combinación de unidades fisicoquímicas permite una notable eliminación de sólidos totales, resultando en una gran disminución de compuestos que inhiben el crecimiento microbiano, turbidez y color, lo que facilita notablemente el posterior cultivo microalgal. Además, tanto *Chlorella pyrenoidosa* como *Scenedesmus obliquus* fueron capaces de crecer en las ARA como medio de cultivo,

obteniendo unos elevados porcentajes de eliminación para diversos parámetros tales como la DQO, COT, CI, NT, etc. La biomasa final obtenida fue rica en carbohidratos, alcanzando valores de hasta el 72,5% (*Scendedesmus obliquus*) y el 89,2% (*Chlorella pyrenoidosa)* y lípidos, con valores máximos del 34,2% (*Chlorella pyrenoidosa)* y 44,9% *(Scenedesmus obliquus).*

Se puede concluir que la combinación de operaciones fisicoquímicas con el cultivo de microalgas constituye un proceso efectivo para el tratamiento de las ARAs, permitiendo la obtención simultanea de agua tratada de alta calidad y biomasa microalgal con alto valor añadido que puede emplearse en la producción de biocombustibles tales como biodiesel y biogás.

Por otro lado, el segundo gran desafío al que se enfrenta la industria oleícola está relacionado con la correcta caracterización y autentificación de los aceites de oliva. Actualmente, la dieta mediterránea es difícil de concebir sin este aceite. Su alto contenido en ácidos grasos monosaturados, vitamina E y antioxidantes lo convierten en uno de los aceites vegetales con mayores beneficios para la salud. Esto, junto con sus atributos organolépticos, ha provocado que el interés y consumo de aceite de oliva de alta calidad se expanda a nivel mundial. En este sentido, la calidad del aceite de oliva viene determinada por su composición química, que depende de numerosos factores y que puede verse alterada por procesos térmicos, de oxidación o por prácticas incorrectas durante la extracción o almacenamiento de este. Actualmente, el Consejo Oleícola Internacional, define la calidad de los aceites de oliva en base a cuatro parámetros: la acidez libre, el índice de peróxidos, los coeficientes de extinción ultravioleta y las características sensoriales. Sin embargo, a medida que aumenta el valor del aceite de oliva, también lo hace el riesgo de que se lleven a cabo malas prácticas tales como su incorrecta caracterización, etiquetado y clasificación dentro de los diferentes tipos de aceite de oliva o su adulteración con otros aceites de menor calidad.

En este trabajo de investigación, se ha propuesto el empleo de tres técnicas alternativas que permiten la correcta evaluación de la calidad nutricional, el estado de conservación y la estabilidad oxidativa de los aceites de oliva. Concretamente, se han estudiado cuatro aceites de oliva virgen extra de diferentes variedades y se han determinado, en primer lugar, el perfil de ácidos grasos mediante cromatografía de gases. Además, se ha empleado la técnica de Calorimetría Diferencial de Barrido (CDB) para estudiar tanto la calidad como la estabilidad oxidativa de los aceites de oliva

cuando estos son sometidos a altas temperaturas. También, mediante espectrofotometría ultravioleta se ha estudiado la presencia de productos primarios y secundarios de la oxidación mediante el cálculo de los coeficientes de extinción ultravioleta (K_{232} y K_{270}).

Los resultados obtenidos revelaron que el ácido oleico fue el más abundante en las cuatro variedades de AOVE estudiadas, con una concentración promedio del 77,1%. Además, la CDB demostró ser un técnica eficiente, rápida, precisa y respetuosa con el medio ambiente que permite tanto la determinación de cambios ocurridos en la composición química del aceite de oliva (a consecuencia de la termo-oxidación) como la determinación de la temperatura de inicio a la oxidación, que permitió confirmar que los cuatro AOVE estudiados poseen una estabilidad termo-oxidativa muy similar. Por último, la determinación de los coeficientes K_{232} y K_{270} permitió descartar la presencia de productos de oxidación y confirmar la correcta clasificación de las muestras como AOVE.

Finalmente, se puede concluir por tanto que la evaluación del perfil de ácidos grasos, la temperatura de inicio a la oxidación y los valores de K_{232} y K_{270}, representan un conjunto de parámetros adecuados, precisos y fáciles de determinar para predecir la calidad, el estado de conservación y la estabilidad oxidativa de los aceites de oliva o los aceites vegetales en general.

ABSTRACT

This Doctoral Thesis has been developed in the Laboratories of the Chemical Engineering Area at Pablo de Olavide University, Seville (Spain). This research work has studied the bioremediation of wastewaters (urban and industrial) by combining physicochemical treatments and microalgae cultures. In addition, new methods to determine the stability of olive oils are proposed.

Spain has the largest olive grove in the world; besides, it is the world leader in production, commercialization and export of olive oils, which results in a great economic, social, environmental, and cultural value. The importance of the olive oil industry is shown by the fact that Spain is responsible for the 60% of the European Union production and 50% of the worldwide. However, this large production has resulted in a series of challenges regarding the management of the wastes generated and the authentication and traceability of olive oils, which must be faced by the olive oil industry.

On the one hand, olive oils production generates large volumes of olive oil mill wastewaters (OMWs). The physicochemical composition of OMWs is characterized by a high heterogeneity and depends on the process used for olive oil extraction. This process can be performed in discontinuous (press) or continuous (by centrifugation) forms. Continuous process can be performed using a 'Decanter' with two (one for olive oil and another one for pomace) or three exits (olive oil, pomace and vegetation water), being the centrifugation system with a two exits 'Decanter' the one used in Spain. OMWs generated through this system are characterized by a slightly acid pH and a high organic matter content, which includes polysaccharides, sugars, organic acids and high concentrations of phenolic compounds (major contributors to OMWs toxicity due to their high phytotoxicity and antimicrobial activity). Nowadays, OMWs accumulation in large reservoirs for water evaporation during the summer months represents the most used management system by olive mills. However, this system leads to numerous problems such as groundwater contamination, bad odours generation and proliferation of insects. For these reasons, and due to the great impact of OMWs on the environment, the treatment of these wastewaters represents a great challenge for the olive oil industry.

This research work proposes a novel bioprocess based on the combination of physicochemical operations with microalgae culture as an integral system for OMWs treatment. The aim of this process is to obtain a high-quality final water that is suitable for reuse in industrial activities, irrigation or direct discharge into natural watercourses. In addition, the obtained microalgal biomass is rich in energetic compounds and therefore, has a great economic value.

To this end, three bioprocesses have been designed and executed and their effectiveness in OMWs treatment and microalgal growth has been studied. The first bioprocess consisted of a primary physicochemical pretreatment (flocculation-sedimentation, UV-photolysis and microfiltration) followed by *Chlorella pyrenoidosa* culture in different concentrations of pretreated OMWs. The second consisted of a primary treatment (flocculation-sedimentation and microfiltration) followed by *Scendesmus obliquus* culture in different pretreated OMWs concentrations. Finally, it was studied the combination of OMW (pretreated by flocculation-sedimentation and UV-photolysis) with urban wastewater, for the simultaneous treatment of both wastewaters and the formation of different culture media for *Chlorella pyrenoidosa* growth. In the three experimental series, OMWs physicochemical characteristics were determined throughout both the primary treatment and the microalgae culture by measuring parameters such as the total phenolic compounds (TPCs), total carbon (TC), total organic carbon (TOC), inorganic carbon (IC), etc. In addition, microalgal kinetic growth was evaluated based on the maximum specific growth rate (μ_m) and volumetric biomass productivity (P_b), the biochemical composition of the final biomass was also determined at the end of each culture.

Results showed that the establishment of a primary treatment, based on the combination of physicochemical units, allows a great elimination of total solids, resulting in the removal of inhibitory compounds, turbidity and colour, which significantly improves the subsequent microalgae culture. In addition, both *Chlorella pyrenoidosa* and *Scenedesmus obliquus* can grow in OMWs as culture medium, reaching high removal percentages for parameters such a COD, TOC, IC, TN, etc. The final biomass obtained was rich in carbohydrates, with values up to 72.5% (*Scendedesmus obliquus*) and 89.2% (*Chlorella pyrenoidosa*) and lipids with maximum values of 34.2% (*Chlorella pyrenoidosa*) and 44.9% (*Scenedesmus obliquus*).

It can be therefore concluded that the combination of physicochemical operations with microalgae cultures constitutes an effective system for OMW treatment, obtaining simultaneously a high quality water and a high added value biomass, which can be used in biofuels production such as biodiesel or biogas.

On the other hand, the second major challenge faced by the olive oil industry is related to the correct characterization and authentication of olive oils. Currently, the Mediterranean diet is difficult to conceive without this oil. Its high content in monosaturated fatty acids, vitamin E and antioxidants make it one of the vegetable oils with the greatest health benefits. This, together with its organoleptic attributes, has resulted in the worldwide expansion of high-quality olive oils consumption. In this sense, olive oil quality is determined by its chemical composition, which depends on numerous factors and can be altered because of thermal processes, oxidation or incorrect practices during the extraction or storage. The International Olive Council defines the quality of olive oil according to four parameters: free acidity, peroxide index, ultraviolet extinction coefficients and sensory characteristics. However, with the rise of the economic value of olive oil, the risk of bad practices such as incorrect characterization, labelling and classification within the different types of olive oil, or adulteration with lower quality oils is augmented.

This research work proposes the use of three alternative techniques, which allow the correct evaluation of the nutritional quality, the conservation status and the oxidative stability of different extra virgin olive oils (EVOO). Precisely, four EVOO from different varieties have been studied. First, fatty acid profiles have been determined by means of gas chromatography. Second, Differential Scanning Calorimetry (DSC) has been used to study both the quality and the oxidative stability of EVOO when it is subjected to high temperatures. Finally, by means of ultraviolet spectrophotometry, the presence of primary and secondary oxidation products has been studied by determining the UV extinction coefficients (K_{232} and K_{270}).

Experimental results revealed that oleic acid was the most abundant in the four EVOO studied, with an average concentration of 77.1%. Furthermore, DSC proved to be an efficient, fast, accurate and environmentally friendly technique that allows both the determination of changes in olive oil chemical composition (as a consequence of thermo-oxidation) and the oxidation onset

temperature, which proved that the four EVOO studied exhibited a very similar thermo-oxidative stability. Finally, the determination of the coefficients K_{232} and K_{270} allowed to confirm the absence of oxidation products as well as the correct classification of the samples as EVOO.

Finally, it can be therefore concluded that the evaluation of the fatty acids profile, the oxidation onset temperature and the K_{232} and K_{270} values, represent a suitable, simple and precise set of parameters to predict the quality, state of conservation and oxidative stability of olive oils or vegetable oils.

1. INTRODUCCIÓN

1.1. LA INDUSTRIA DE LOS ACEITES DE OLIVA

El olivo (*Olea europaea L)* es un árbol tradicionalmente cultivado para la producción de aceite de oliva y aceitunas. Más del 97% de los olivos que se cultivan actualmente a nivel mundial se localizan en la cuenca del mar Mediterráneo, siendo España el principal productor mundial con una producción de aceites de oliva de $1{,}79\times10^6$ toneladas en la campaña 2018/2019 según el Consejo Oleícola Internacional. Le siguen Italia, Grecia, Turquía, Túnez, Marruecos, Portugal y Siria como principales países productores (FAOSTAT, 2015; COI, 2019).

Según el Consejo Oleícola Internacional, la producción mundial de aceites de oliva en la campaña 2018/2019 ha sido de $3{,}22\times10^6$ toneladas. Una apreciable proporción del 70,3% se debe a la producción europea que alberga a los tres mayores productores de aceite de oliva: España con una producción del 55,6%, Grecia que produjo el 5,75% e Italia con el 5,4% (COI, 2019).

Durante la última década (2009-2019) la producción de aceite de oliva ha aumentado en torno a un 10% mundialmente respecto a la década anterior (1999-2009), proviniendo de España la mayor contribución a dicho aumento, donde la producción de aceite de oliva ha experimentado un aumento del 27% en el mismo periodo (COI, 2019).

1.1.1. Los aceites de oliva

El aceite de oliva es un aceite vegetal obtenido de las aceitunas mediante procedimientos exclusivamente mecánicos. Su extracción se realiza mediante una serie de etapas que incluyen la recogida, lavado y trituración de la aceituna, batido, centrifugación, almacenamiento y filtración (Di Giovacchino et al., 2002). Las propiedades del aceite de oliva están determinadas por su composición química, así como el estado de las aceitunas. En este sentido, para la producción de aceites de alta calidad, las aceitunas deben recolectarse sin romper la piel y se deben procesar en un plazo de menos de 24 horas desde la recolección (Calabriso et al., 2015). Actualmente, en algunos casos, el proceso de eleboración se lleva a cabo a los 30 minutos de la recolección. Además, la extracción debe realizarse a partir de frutos sanos, evitando manipulaciones o tratamiento que puedan alterar la composición química del aceite de oliva durante el proceso de extracción y almacenamiento (Oliveras López, 2005).

A pesar de que existen numerosas formas para definir la calidad del aceite de oliva, el Consejo Oleícola Internacional y el Reglamento de la Comisión han definido la calidad del aceite de oliva considerando cuatro parámetros: el contenido en ácidos grasos libres, el índice de peróxidos, los coeficientes de extinción específicos UV (K_{232} y K_{270}) y la puntuación sensorial. La clasificación general, según el COI y el CODEX ALIMENTARIUS, divide los aceites de oliva en ocho categorías comerciales: aceite de oliva virgen extra, aceite de oliva virgen, aceite de oliva corriente, aceite de oliva lampante, aceite de oliva refinado, aceite de oliva, aceite de orujo crudo, aceite de orujo refinado y aceite de orujo de oliva. La clasificación de la Unión Europea es más restrictiva y solo considera siete categorías, no incluye la categoría 'aceite de oliva corriente'. Esta clasificación general se basa en los atributos sensoriales (sabor y aroma) y el contenido de ácidos grasos libres, sin embargo, no incluye requisitos relevantes como la estabilidad a la oxidación o el contenido fenólico (Kalua et al., 2007).

1.1.2. Criterios de calidad

Los criterios de calidad del aceite de oliva se basan en su composición química y en la valoración sensorial de sus propiedades organolépticas, que deben ser definidos por un panel de expertos perfectamente entrenado y acreditado.

1.1.2.1. *Acidez libre*

Se define como el contenido de ácidos grasos libres expresado en porcentaje de ácido oleico. Las grasas producidas biológicamente son neutras, lo que significa que el aceite procedente de aceitunas en buen estado y de buena calidad tiene un 0% de acidez libre. Por lo tanto, la presencia de ácidos grasos libres es una anomalía resultante del mal estado de los frutos, así como procesos inadecuados de tratamiento y conservación (Guzmán et al., 2015).

Este parámetro se calcula mediante el método convencional de valoración, que consiste en disolver la muestra en una mezcla de disolventes y medir los ácidos grasos libres mediante análisis volumétrico utilizando una disolución etanólica de hidróxido de potasio (Guzmán et al., 2015).

1.1.2.2. *Índice de peróxidos*

Mide el estado de oxidación inicial de un aceite, expresado en miliequivalentes de oxígeno activo por kilogramo de grasa. Las grasas se oxidan cuando entran en contacto con oxígeno, dando lugar a la formación de varios compuestos como los peróxidos, considerados los primeros productos de oxidación. Además, este parámetro también indica la degradación de determinados componentes de interés nutricional, como la vitamina E (Guzmán et al., 2015).

Este parámetro se mide disolviendo la muestra en ácido acético y cloroformo, posteriormente se trata con una solución de yoduro de potasio y el yodo liberado si titula con una disolución de tiosulfato de sodio (Guzmán et al., 2015).

1.1.2.3. *Absorbancia en el ultravioleta*

Los valores de K_{232} y K_{270} son medidas espectrofotométricas para cuantificar la absorción al UV a 232 y 270 nm. Proporcionan información sobre la calidad del aceite, el estado de conservación y cualquier deterioro que se produzco durante los procesos tecnológicos. K_{232} mide la fase inicial de oxidación del aceite de oliva y K_{270} indica estados oxidativos avanzados, puesto que el contenido en peróxidos va cambiando a medida que el proceso de oxidación ocurre (Guzmán et al., 2015).

Para su medida se disuelve la muestra en ciclohexano y se mide la absorbancia de la disolución a las longitudes de onda mencionadas.

1.1.2.4. *Valoración sensorial*

Las características sensoriales del aceite de oliva se deben a los atributos visuales, olfativos y gustativos, determinados por la presencia de componentes que contribuyen a cada uno de ellos (Peri, 2014).

1.2. PROCESO DE EXTRACCIÓN DEL ACEITE DE OLIVA

El sistema de elaboración del aceite de oliva ha evolucionado a lo largo de la historia debido a razones ambientales y económicas. Actualmente, existen dos sistemas de extracción: el sistema tradicional de prensa, empleado durante siglos por las fábricas de aceite, y el sistema de centrifugación, adoptado por la industria del aceite de oliva durante las últimas décadas. Por otra parte, existen dos métodos dentro del sistema de centrifugación: los sistemas con decánter de tres y dos salidas (Roig et al., 2006).

1.2.1. Sistema tradicional de prensa

Durante el sistema tradicional de prensa, en primer lugar, se realiza la separación de las hojas y el lavado de las aceitunas para a continuación, llevar a cabo la molienda de las mismas en molinos con el objetivo de extraer la mayor cantidad de aceite contenido en las vacuolas de las células del mesocarpio. Dicha etapa tiene una duración de entre 20 y 30 minutos y durante la misma se produce un ligero aumento de la temperatura (3-5ºC) debido a la baja velocidad de rotación que se emplea (12-15 rpm), esto evita la formación de emulsiones e incrementa el rendimiento de la extracción (Di Giovacchino, 2013). El producto que se obtiene al final de esta etapa es una pasta compuesta por agua de vegetación, aceite y partículas sólidas procedentes del hueso y de la piel de la aceituna (Oliveras-López, 2005).

La pasta obtenida tras la molienda debe someterse a un proceso de batido para conseguir un mayor rendimiento de extracción, siendo esta etapa un proceso lento de agitación continua que aumenta la cantidad de aceite extraído mediante la formación de gotas de mayor tamaño, evitando la formación de emulsiones agua/aceite. Esta etapa de batido tiene una duración de entre 20 y 30 minutos y durante la misma, la pasta de aceitunas es calentada, hasta una temperatura que no debe exceder los 22-25 °C, mediante la circulación de agua caliente a través de una camisa de termostatación (Di Giovacchino, 2013).

Por último, para la separación del aceite de oliva del resto de componentes se empleaban tradicionalmente discos o capachos de fibra fabricados con fibras de esparto que actualmente han sido sustituidos por discos de fibra sintética (nylon y coco) que permiten una limpieza y

mantenimiento más sencillo. Durante este sistema se aplica presión sobre los discos para llevar a cabo la compactación de la fase sólida y la separación de las fases líquidas: aceite de oliva y agua de vegetación. Al final, en esta etapa es añadida una pequeña cantidad de agua que facilita la separación del aceite de las otras fases. Una vez terminado el proceso queda una fracción sólida denominada orujo (compuesta por la pulpa, piel, hueso y agua de las aceitunas) y una fracción liquida (aceite, agua y partículas en pequeña proporción) que es posteriormente separada mediante decantación o centrifugación del agua residual generada en el proceso (Dermeche et al., 2013).

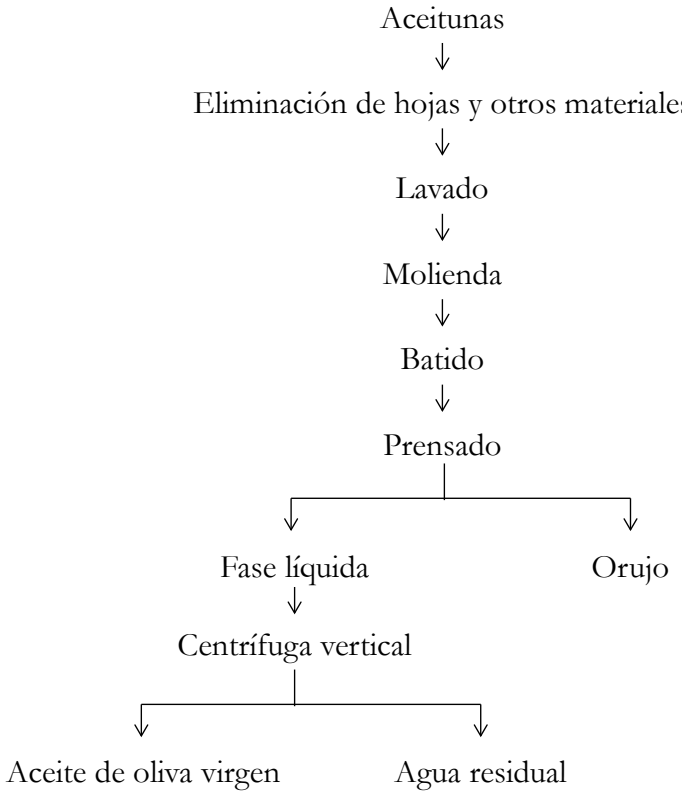

Figura 1. Diagrama del proceso de extracción de aceite de oliva mediante el sistema de prensado. Adaptado de Di Giovacchino, 2013.

El empleo de este sistema de extracción presenta determinadas ventajas como la simplicidad de la maquinaria requerida y el menor consumo de energía eléctrica. En cuanto a los residuos generados, la cantidad de agua residual producida es muy baja, caracterizándose por tener

una escasa cantidad de aceite en su composición. En cuanto a los inconvenientes que presenta, la maquinaria empleada requiere mayor mano de obra y limpieza. Además, el empleo de molinos es una tecnología lenta, con una baja capacidad de carga y no permite la operación en continuo (Di Giovacchino, 2013). A veces, en frutos sanos y de árbol, se evita la operación de lavado de la aceituna.

1.2.2. Sistema de centrifugación

Este método de extracción se basa en el empleo de decantadores industriales para separar, tras el batido, el aceite de oliva de la fracción líquida (agua de vegetación) y de la fracción sólida (el orujo) por diferencia de densidad mediante la aplicación de fuerza centrífuga (Oliveras-López, 2005).

Las dos primeras etapas del proceso de centrifugación consisten en la eliminación de las hojas de aceitunas y su posterior lavado (en el caso de que sea necesario). Ambas etapas son de vital importancia puesto que ayudan a evitar daños y atascos en las posteriores etapas. Una vez se han llevado a cabo, la trituración de las aceitunas puede realizarse empleando diferentes tipos de molinos: martillo, discos dentados o cilindros estriados. Todos ellos se basan en una estructura metálica que gira a elevada velocidad provocando el choque de las aceitunas contra una reja metálica dando lugar a las pastas de aceitunas (Di Giovacchino, 2013).

La siguiente etapa del proceso consiste en el batido de la pasta obtenida para la formación de gotas de aceite de mayor tamaño mediante la unión de gotas más pequeñas. Además, esta etapa permite eliminar la rotura de células que no han sido trituradas y que contienen aceite en su interior. Esta etapa puede llevarse a cabo en diferentes tiempos y a diferentes temperaturas, pero en general, suele tener una duración de entre 30 y 90 minutos y la temperatura varía entre 25 °C y 32 °C (Di Giovacchino, 2013).

El proceso de separación del aceite del resto de fases es llevado a cabo por una centrífuga horizontal que rota a elevada velocidad, generalmente entre 2800 y 3500 rpm, permitiendo la extracción del aceite de oliva mediante la operación en continuo (Di Giovacchino, 2013).

Dentro de este sistema se encuentra el sistema de centrifugación con decánter de tres salidas ampliamente utilizado durante los años 70 y 80 y que sigue en uso actualmente pero que ha sido parcialmente sustituido, y el sistema de centrifugación con decánter de dos salidas. La principal diferencia entre ambos sistemas es la reducción, en el decánter de dos salidas, de la cantidad de agua que se añade para llevar a cabo la separación del aceite del resto de los componentes, lo cual tiene un efecto directo tanto en la composición del aceite extraído como en la capacidad de carga del decánter (Di Giovacchino, 2013).

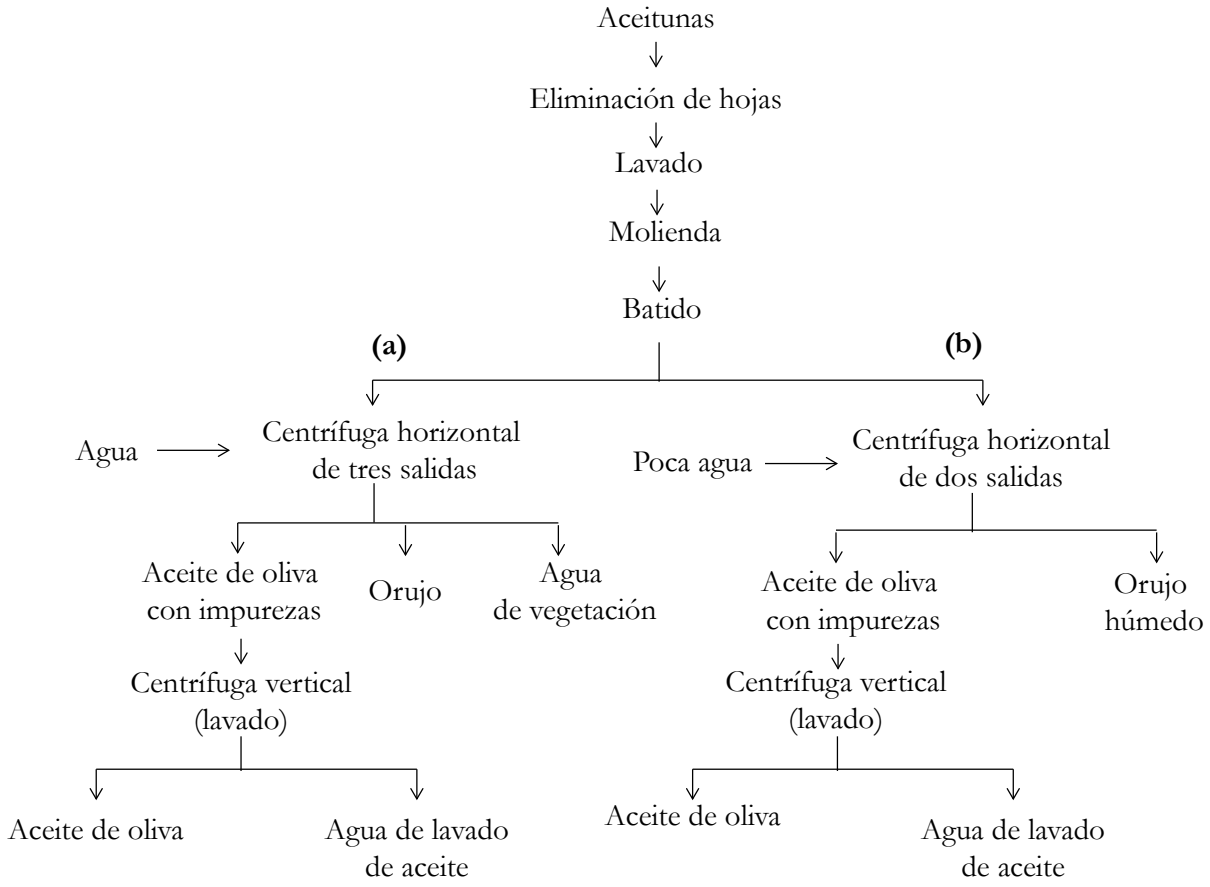

Figura 2. Diagrama del proceso de extracción de aceite de oliva mediante el sistema de centrifugación: sistema de extracción con decánter de tres salidas (a) y sistema de extracción con decánter de dos salidas (b).

1.2.2.1. Sistema de extracción con decánter de tres salidas.

En el sistema de extracción con decánter de tres salidas, una vez se ha llevado a cabo la molienda y el batido de la pasta, ésta es bombeada al decánter mediante la adición de agua caliente para aumentar la fluidez de la misma y facilitar la separación de las fases liquidas y sólidas mediante centrifugación. Tras la centrifugación se obtienen tres corrientes de salida: una sólida (orujo con agua y algo de aceite) y dos líquidas (aceite de oliva y agua residual), Di Giovacchino, 2013.

Este sistema presenta diversas ventajas respecto al prensado tradicional: automatización, mejor calidad del aceite y menor área requerida, también tiene una serie de inconvenientes si es comparado con el sistema de extracción de dos fases (Roig et al., 2006).

1.2.2.2. Sistema de extracción con decánter de dos salidas.

En este sistema la adición de agua del exterior para la separación del aceite de las demás fases es algo menor en comparación con el sistema que utiliza el decánter de tres salidas, por lo que el volumen de agua residual que se genera puede ser más pequeño (Oliveras-López, 2005).

Tras la centrifugación se obtienen dos corrientes de salida: una oleosa y una semisólida (similar a un lodo) con bastante humedad (Oliveras-López, 2005).

Inicialmente, la principal ventaja de este sistema es el menor consumo de agua potable en el decánter, aunque en la centrífuga vertical es necesario incrementar el consumo de agua. Por otra parte, el rendimiento de extracción de la pasta puede ser menor por lo que supone un mayor gasto económico. Además, la elevada humedad del orujo dificulta su manipulación (Oliveras-López, 2005).

1.3. SUBPRODUCTOS Y RESIDUOS GENERADOS

El proceso de extracción del aceite de oliva supone un importante impacto ambiental debido a las elevadas cantidades de subproductos y residuos que se generan durante el mismo. Dichos residuos son de dos tipos: líquidos (aguas residuales de almazara, ARA) y sólidos (orujos y fragmentos de huesos de aceitunas). El aprovechamiento de los subproductos y el tratamiento de los residuos es uno de los principales problemas a los que se enfrentan actualmente la industria de los aceites de oliva (Christoforou y Fokaides, 2016).

En cuanto a los residuos líquidos, la cantidad de agua residual que se genera en el proceso de producción del aceite de oliva en el área del Mediterráneo se estima en torno a 1,2 millones de toneladas al año. Sin embargo, este valor varía en función del sistema de extracción empleado. La implementación del sistema de centrifugación con decánter de dos salidas en el 90% de las almazaras españolas ha permitido una reducción en el consumo de agua y, por lo tanto, en la generación de aguas residuales (Borja et al., 2006). Las aguas residuales generadas durante dicho sistema de extracción son una mezcla de aguas procedentes del lavado de las aceitunas antes de la etapa de molienda y de las aguas de lavado del aceite en la centrífuga vertical. La cantidad generada de estas aguas se estima en 250 litros por cada tonelada de aceitunas (Borja et al., 2006). Por otro lado, las ARA generadas durante el proceso de extracción con decánter de tres salidas se componen de una mezcla de las aguas generadas durante el lavado de aceituna y el agua resultante de la etapa de lavado de aceite, generándose una cantidad de entre 60 y 100 litros por cada 100 kg de aceitunas (Di Giovacchino, 2013).

En cuanto a los subproductos, durante el sistema de extracción con decánter de tres salidas se genera el orujo, compuesto principalmente por pulpa seca y huesos de aceitunas. Por cada tonelada de aceitunas que se someten al proceso, se generan alrededor de 550 kg de orujo (Rincón et al., 2012). Por otro lado, el sistema de extracción con decánter de dos salidas da lugar a un orujo muy húmedo, compuesto por pulpa, agua, semillas y huesos procedentes de las aceitunas. Por cada tonelada de aceituna que se procesa se generan alrededor de 800 kg de orujos (Ballesteros et al., 2001).

1.3.1. Subproductos sólidos

1.3.1.1. Características fisicoquímicas

i. Orujo

La composición fisicoquímica del orujo varía en función de la variedad y el origen de las aceitunas, las condiciones de cultivo y el proceso de extracción de aceite de oliva empleado (prensa o centrifugación de tres fases). La celulosa, hemicelulosa y lignina son los componentes más abundantes del mismo, pudiéndose encontrar además elevadas concentraciones de lípidos y proteínas. Se caracteriza además por tener un contenido de humedad que varía entre el 25-35%, en el caso del orujo obtenido mediante el sistema de prensa, o del 45-55%, en el sistema de centrifugación con decánter de tres salidas. Además, tiene un contenido en cenizas entre el 1,7-4,0%. En cuanto a su composición mineral, el compuesto más abundante es el potasio, seguido del calcio y sodio. La Tabla 1 recoge las principales características químicas del orujo (Dermeche et al., 2013).

ii. Orujos de procesos con decánter de dos salidas

Es un subproducto sólido/líquido generado durante el sistema de extracción. Aproximadamente entre 35 y 40 kg de orujo es generado por cada 100 kg de aceitunas que son sometidas al proceso. Este subproducto está formado por una masa heterogénea con un alto contenido tanto en agua como en aceite (Nunes et al., 2016).

Se compone de fragmentos de la piel (pericarpio), pulpa (mesocarpio) y hueso (endocarpio) procedentes de las aceitunas, así como agua de vegetación (Nunes et al., 2016). Su contenido en cenizas varía entre el 1,4-4,0% y su contenido en agua entre el 65-75%. En cuanto a su contenido en materia orgánica, éste varía entre el 60-98%, siendo la lignina, hemicelulosa y celulosa los principales componentes que se encuentran en el mismo. Otros compuestos orgánicos presentes son los lípidos, carbohidratos y proteínas. En cuanto a su composición mineral, el elemento mayoritario es el potasio, seguido de calcio y sodio. Otro rasgo que caracteriza la composición de este residuo es la presencia de elevadas concentraciones de

compuestos fenólicos, los cuales en parte se encuentran disueltos en la fracción acuosa del mismo (Dermeche et al., 2013).

Tabla 1. Características químicas de los orujos procedentes de procesos con decánter de tres salidas (Orujos 3S) y de dos salidas (Orujos 2S). Adaptado de Dermeche et al., 2013.

Compuesto	**Orujos 3S**	**Orujos 2S**
Pulpa, %	12 - 35	10 - 15
Hueso de aceituna, %	15 - 45	12 – 18
Peso seco, %	87,1 - 94,4	
Ceniza, %	1,7 - 4	1,42 – 4
Carbono total, %	29,03 - 42,9	25
Materia orgánica, %	85	60,3 – 98,5
Nitrógeno total, %	0,2 - 0,3	0,25 – 1,85
Fósforo, %	0,03 - 0,06	0,03 – 0,14
Potasio, %	0,1 - 0,2	0,63 – 2,9
Lípidos	3,5 - 8,72	3,76 – 18
Compuestos fenólicos totales	0,2 - 1,15	0,4 – 2,43
Azúcares totales	0,99 - 1,38	0,83 – 19,3
Proteínas totales	3,43 - 7,26	2,87 – 7,2
Celulosa	17,37 - 24,14	14,5
Hemicelulosa	7,91 - 11,00	6,63
Lignina	0,21 - 14,18	8,54

1.3.1.2. Aplicaciones

i. Producción de biocombustibles

– Bioetanol. La elevada concentración de materia orgánica presente en los orujos los convierte en una potencial fuente para la producción de etanol. El proceso se lleva a cabo en dos etapas que consisten en un pretratamiento, destinado a la liberación de los azúcares presentes, y en

segundo lugar se lleva a cabo la conversión de dichos azúcares en etanol que podría ser llevado a cabo por levaduras (Dermeche et al., 2013).

- Biometano. La producción de metano a partir de los residuos sólidos del aceite de oliva se lleva a cabo mediante un proceso que consta de dos etapas. En primer lugar, se realiza un pretratamiento que permite la posterior obtención de un mayor rendimiento de metano. En segundo lugar, se realiza un proceso de digestión anaerobia. En este sentido, estudios recientes han demostrado que la digestión anaerobia de dos etapas obtiene mayores rendimientos que la convencional en una etapa. En la primera etapa, la materia orgánica compleja es transformada en compuestos intermediarios, tales como ácidos grasos volátiles y alcoholes, por bacterias acidogénicas. En la segunda, estos intermediarios son convertidos en CH_4 y CO_2 por organismos metanógenos o arqueas (Dermeche et al., 2013; Fezzani y Cheikh, 2010).

- Biodiesel. Es un biocombustible renovable, biodegradable y cuya producción genera escasas emisiones de CO_2 y NO_X. Esto lo convierte en uno de los biocombustibles más prometedores y respetuosos con el medio ambiente (Hernández et al., 2014). Su producción se lleva a cabo mediante una reacción de transesterificación de lípidos con alcoholes de cadena corta (Atadashi et al., 2012).

  Por un lado, el empleo de una lipasa de *Thermomyces lanuginosus* inmovilizada sobre orujo previamente activado con poliglutaraldehído permite la obtención de un rendimiento de hasta el 93% en la producción de biodiesel. Además, el orujo como material de soporte presenta la doble ventaja de ser un material renovable de bajo coste, lo que permite su reutilización (Yücel, 2011). Por otro lado, se ha demostrado que los lípidos presentes en los orujos procedentes de procesos con decánter de dos salidas son una alternativa viable para la producción de biodiesel, llegando a alcanzar elevados índices de conversión de hasta el 94,7% (Hernández et al., 2014).

- Biohidrógeno. La producción de biohidrógeno (bio-H_2) mediante fermentación oscura es un tipo de digestión anaerobia que consiste en una etapa de hidrólisis seguida de una de acidogénesis, dando lugar a hidrógeno, dióxido de carbono y compuestos orgánicos simples tales como ácidos grasos volátiles y alcoholes (Rincón et al., 2012). Además, los microorganismos fotosintéticos han despertado especial interés debido a su capacidad de convertir de forma directa la energía solar en bio-H_2 a partir de sustratos tanto orgánicos como inorgánicos. Es por ello que los diferentes tipos de orujos han demostrado ser sustratos efectivos para ambos procesos, obteniéndose mayores rendimientos (en el caso de la producción mediante fotosíntesis) cuando son diluidos con agua, debido a su color oscuro (Dermeche et al., 2013).

ii. Obtención de compuestos bioactivos

Ambos tipos de orujos son ricos en una gran variedad de nutrientes procedentes de las aceitunas y del aceite residual. Entre ellos destacan los compuestos fenólicos, con numerosas propiedades antioxidantes, antiinflamatorias y antimicrobianas, siendo el tirosol e hidroxitirosol los más abundantes. Otros compuestos de alto valor añadido que se encuentran en cantidades significativas son la oleuropeína, el ácido cafeico, verbascósidos, el ácido oleanólico y el catecol, entre otros muchos. La extracción de dichos compuestos para su posterior aplicación en numerosos ámbitos ha sido ampliamente estudiada: campos eléctricos pulsados, descargas eléctricas de alto voltaje o la extracción mediante ultrasonido son algunas de las técnicas que se han estudiado para la extracción de los compuestos fenólicos y proteínas presentes en este residuo (Nunes et al. 2016).

iii. Extracción del aceite residual

La aplicación más extendida del orujo es la extracción del aceite de oliva residual contenido en el mismo, cuya concentración se sitúa en el rango 1,5-3,0% del peso húmedo y puede ser extraído mediante tratamientos químicos y mecánicos. El método más extendido se basa en una primera etapa de centrifugación, en la que se extrae entre el 40% y el 50% del aceite contenido, seguida de una etapa de secado (400-800ºC) en la que se reduce la humedad desde el 60-70% a un

8-12% (Humedad Relativa de Equilibrio). Por último, se realiza una extracción con hexano técnico. El orujo extractado es empleado para la cogeneración de calor y electricidad, energía que a su vez es usada por las extractoras de orujos para llevar a cabo el proceso de secado (Rincón et al., 2012).

Por otro lado, los orujos procedentes del sistema de centrifugación con decánter de dos salidas, con un 3,5% (en base al peso húmedo) de aceite residual en su composición también es empleado para la extracción del aceite residual. Sin embargo, debido a la mayor humedad del mismo, tanto la intensidad como la duración de la etapa de secado deben ser mayores. Además, el agua de vegetación contenida en estos orujos así como la alta concentración de azúcares reductores le aportan una consistencia pastosa que dificulta el secado del mismo (Rincón et al., 2012).

iv. Producción de enzimas

La producción de enzimas industriales puede ser llevada a cabo tanto por levaduras como por hongos filamentosos empleando los orujos como sustrato. Las principales enzimas obtenidas son lipasas mediante el cultivo de *Rhizomucor pusillus* y *Rhizopus rhizopodiformis* (Dermeche et al., 2013).

1.3.1.3. Problemática ambiental

Uno de los principales problemas derivados de la producción de aceites de oliva es la generación de grandes cantidades de orujos en periodos cortos de tiempo. Esto provoca que las almazaras no dispongan de la capacidad suficiente para procesar dichos subproductos durante la temporada de recolección de la aceituna (Romero et al., 2013). Además, el fuerte impacto negativo que tienen sobre el suelo impide su uso directo con fines agrícolas. Estos efectos se deben principalmente a su elevado contenido en compuesto fenólicos, compuestos lipídicos, ácidos orgánicos, bajo pH, salinidad, etc. Todo ello les aporta un alto poder fitotóxico y antimicrobiano. Además, la contaminación fúngica de los mismos provoca que las toxinas procedentes de los hongos combinadas con los compuestos fenólicos sean resistentes a la degradación bacteriana, convirtiéndose en una importante fuente de contaminación (Lammi et al., 2019).

Además de lo anteriormente mencionado, ambos subproductos se caracterizan por un fuerte olor, suponiendo un serio problema para las almazaras y el entorno de las mismas. Esto se debe al ácido pentanoico y al 4-etilfenol; este último es un compuesto lipófilo que se acumula en la fracción oleosa (Romero et al., 2013).

Por otro lado, la consistencia pastosa de los orujos procedentes de procesos con decánter de dos salidas dificulta su transporte, almacenamiento y manipulación, por lo que requiere de unas instalaciones específicas tales como tanques de almacenamiento, bombas de masa y camiones con carrocerías especiales o bien cisternas (Borja et al., 2006; García et al., 2020).

1.3.2. Efluentes líquidos: Aguas residuales de almazara

1.3.2.1. Características fisicoquímicas

Las características fisicoquímicas de las ARA dependen principalmente del método de extracción empleado. Además, otros factores que influyen son las condiciones de cultivo, el tiempo de cosecha, el estado de maduración de las aceitunas o las condiciones climáticas (Dermeche et al., 2013).

En general, las ARA se caracterizan por presentar un color oscuro entre violeta y negro, un fuerte olor a aceite de oliva y un valor de pH que se encuentra entre 3,0 y 6,5. Además, poseen una alta conductividad eléctrica, así como un elevado contenido en compuestos fenólicos y materia orgánica (Borja et al., 2006).

La principal diferencia entre las ARA generadas por los diferentes procesos es la carga orgánica que poseen. En este sentido, el contenido en materia orgánica de las ARA procedentes del sistema con decánter de dos salidas puede ser hasta treinta veces menor que aquel de las ARA procedentes de los otros procesos. Por ello, aunque posean una composición cualitativa similar, la concentración de carga orgánica es mucho menor debido a que la mayoría de los compuestos orgánicos procedentes del agua de vegetación se quedan en los orujos (Borja et al., 2006). Las ARA generadas mediante el sistema de prensa y de centrifugación con decánter de tres salidas presentan valores de DQO y DBO_5 de entre 40-220 y 35-110 g O_2/L, respectivamente. Por otro

lado, las ARA generadas durante el proceso con decánter de dos Salidas presentan valores de DQO y DBO_5 igual a 0,5-65 y 8,5-19 g O_2/L, respectivamente. La materia orgánica incluye azúcares, proteínas, compuestos fenólicos, polialcoholes, pectinas, lípidos, etc.

El elevado contenido en compuestos fenólicos en las ARA se debe a la mayor solubilidad de los mismos en la fase acuosa que en la fase oleosa. Por lo tanto, la mayoría de estos compuestos pasan de la pulpa de las aceitunas a las aguas residuales durante la extracción del aceite de oliva (El-Abbassi et al., 2017). Dentro de los compuestos fenólicos, están muy presentes aquellos que son de bajo peso molecular tales como el hidroxitirosol, tirosol, ácido cafeico y ácido p-cumárico (García y Hodaifa, 2017). Por otro lado, también se encuentran compuestos fenólicos resultantes de la polimerización y autooxidación de los compuestos fenólicos de bajo peso molecular. La presencia de estos compuestos orgánicos recalcitrantes constituye uno de los mayores obstáculos en el tratamiento de las aguas, además, algunos de estos derivados fenólicos son los causantes de la fitotoxicidad de estas aguas (Borja et al., 2006).

En cuanto al contenido en compuestos inorgánicos, el elemento mayoritario es el potasio, seguido del calcio y del sodio (Souilem et al., 2006).

Respecto a las características microbiológicas, las ARA presentan una gran diversidad de microorganismos en su composición, esencialmente bacterias, hongos y levaduras. Las bacterias más presentes pertenecen a los grupos de *Alphaproteobacteria*, *Gammaproteobacteria*, *Betaproteobacteria*, *Firmicutes* y *Actinobacteria*. Además, se han encontrado numerosos agentes infecciosos tales como *Acinetobacter*, *Enterobacter* spp., y *Pseudomonas*. Las levaduras son los microorganismos más abundantes en las ARA, siendo géneros como *Pichia*, *Candida* y *Saccharomyces* las que se encuentran más presentes. Respecto al contenido en hongos, se han detectado hasta 106 especies diferentes pertenecientes a diversos géneros tales como *Acremonium*, *Alternaria*, *Aspergillus*, *Bionectria*, *Byssochlamys*, *Chalara*, *Cerrena*, *Fusarium*, *Lasiodiplodia*, *Lecythophora*, *Paecilomyces*, y *Penicillium*. El papel de dichos hongos es de vital importancia puesto que tienen la capacidad de desintoxicar las ARA mediante la degradación de compuestos fenólicos (El-Abbassi et al., 2017).

1.3.2.2. Aplicaciones

i. Producción de energía y biocombustibles

Las ARA constituyen un buen sustrato para la producción de biohidrógeno, biometano y bioetanol debido a su composición rica en azúcares, ácidos volátiles, polialcoholes y grasas (Dermeche et al., 2013).

En primero lugar, las ARA con un bajo contenido en nitrógeno constituyen un buen sustrato para la producción de biohidrógeno mediante fotofermentación, puesto que concentraciones altas de NH_4^+ provoca la inhibición de la síntesis y actividad de las nitrogenasas (Dermeche et al., 2013).

Por otro lado, numerosos estudios han obtenido elevados rendimientos de biometano empleando ARA como sustrato en la digestión anaerobia. Además, el pretratamiento de las ARA da lugar a la obtención de hasta el doble de este biocombustible. Algunos de los pretratamientos empleados se basan en el uso de hongos como *Aspergillus niger* o *Aspergillus terreus* o levaduras como *Candida tropicalis*. Dichos pretratamientos dan lugar a una reducción de la DQO y de los compuestos fenólicos de las ARA (Dermeche et al., 2013).

Por último, en cuanto a la producción de bioetanol, ésta se lleva a cabo mediante un proceso anaeróbico en el que los carbohidratos presentes en las ARA son convertidos a etanol mediante una serie de etapas. En primer lugar, hay una etapa de pretratamiento seguida de una hidrólisis enzimática y fermentación, también puede llevarse a cabo mediante una sacarificación y fermentación simultáneas (Christoforou y Fokaides, 2016).

ii. Extracción de compuestos de alto valor añadido

Las ARA se caracterizan por contener numerosos compuestos de alto valor añadido tales como sustancias antioxidantes (tirosol, hidroxitirosol y oleuropeína) y otros componentes menores que pueden ser posteriormente empleados en diversas industrias tales como la farmacéutica o cosmética (Roig et al., 2006).

Además, diversos autores han demostrado la idoneidad de las ARA para la producción de biopolímeros tales como el xantano, pululano o polihidroxialcanoatos mediante tratamientos microbiológicos. Además, el tratamiento aeróbico con microorganismos tiene la doble ventaja de permitir la eliminación de compuestos presentes en las ARA responsables de la fitotoxicidad de las mismas. Algunos de los microorganismos que han sido empleados con dicho fin son los hongos *Pleurotus ostreatus, Bacillus pumilus* o la especie de levadura *Yarrowia lipolytica* (Roig et al., 2006).

iii. Compostaje

El compostaje es una de las principales aplicaciones de las ARA puesto que permite su uso como fertilizante evitando los efectos negativos que causa su vertido directo en los campos de cultivo. Previo al proceso de compostaje es necesario que las ARA sean adsorbidas por un sustrato sólido como pueden ser residuos lignocelulósicos. Diversos autores han demostrado los numerosos efectos beneficiosos que reporta para los campos de cultivo la adición de aguas residuales de almazara (Roig et al., 2006).

1.3.2.3. Problemática ambiental

Las ARA son el residuo más abundante generado durante el proceso de extracción del aceite de oliva, siendo consideradas una de las aguas residuales más contaminantes procedentes de la industria agroalimentaria. En la campaña 2014/2015 se produjeron 5,4 x 10^6 m^3 de ARA, según el Consejo Oleícola Internacional, siendo España responsable del 20% (García y Hodaifa, 2017).

Las ARA constituyen un serio problema medioambiental debido principalmente a su elevada demanda química de oxígeno (DQO), así como elevada concentración de compuestos inhibitorios del crecimiento microbiano tales como compuestos fenólicos. Además de los polisacáridos, lípidos, proteínas y compuestos aromáticos, todos ellos inhibidores del crecimiento de poblaciones microbianas anaeróbicas. Por estos motivos y por su elevada toxicidad, fuerte olor, la amenaza que suponen tanto para aguas subterráneas como superficiales y la falta de técnicas apropiadas para su tratamiento, las ARA son una importante fuente de contaminación ambiental, sobre todo para los principales países productores de aceites de oliva (Sabbah, 2009). Además, las

elevadas cantidades que se generan en un breve periodo de tiempo agravan los daños ambientales entre los meses de octubre y marzo en los países del mediterráneo productores de aceites de oliva (Dermeche et al., 2013).

i. Contaminación del aire

El tratamiento más extendido actualmente de las aguas residuales de almazara es su almacenamiento en estanques abiertos para su evaporación (balsas de estabilización). Sin embargo, además de la ocupación de numerosas hectáreas de terreno, esta práctica da lugar a la generación de malos olores como consecuencia de la emisión de metano y otros gases como sulfuro de hidrógeno, procedentes de procesos de fermentación (Souilem et al., 2006).

ii. Contaminación de aguas

Además de los problemas anteriores, el almacenamiento de las ARA en estanques genera numerosos problemas de contaminación de aguas subterráneas y acuíferos por infiltración (García y Hodaifa, 2017). Por otra parte, el vertido directo de las ARA a lagos, ríos o pantanos tiene un efecto negativo directo sobre el ecosistema en el que son vertidas. En primer lugar, la elevada concentración de azúcares y materia orgánica de estas aguas provoca una disminución en la disponibilidad de oxígeno debido a la estimulación del crecimiento microbiano. Además, aguas con un elevado contenido en fósforo son desencadenantes de procesos de eutrofización, respuesta de ecosistemas acuáticos a la adición de nutrientes. Otro impacto negativo de las ARA sobre las aguas superficiales es la coloración de estas debido a su elevado contenido en compuestos fenólicos. Además, los lípidos presentes en las ARA pueden formar una capa impenetrable en la superficie de las aguas en las que son vertidas impidiendo el paso de la luz y el oxígeno molecular y por tanto, inhibiendo el crecimiento vegetal (Dermeche et al., 2013).

Además del impacto negativo sobre ecosistemas de agua dulce, diversos autores han demostrado las alteraciones patológicas que éstas tienen sobre organismos marinos, así como el efecto tóxico de las mismas en comunidades acuáticas (Dermeche et al., 2013).

iii. Contaminación del suelo

Los efectos negativos que tiene el vertido directo de las ARA en suelos y campos de cultivo están asociados a su elevado contenido en sales minerales, bajo pH y a la presencia de compuestos fitotóxicos, especialmente compuestos fenólicos (Roig et al., 2006). Su vertido directo tiene efectos negativos tanto para el crecimiento vegetal y microbiano como para las propiedades fisicoquímicas del suelo, debido principalmente a su contenido en compuestos fenólicos. Además, los compuestos lipídicos presentes en las mismas aumentan la hidrofobicidad de los suelos y disminuyen la capacidad de retención de los mismos (Dermeche et al., 2013).

1.4. AGUAS RESIDUALES URBANAS

1.4.1. Origen

Las aguas residuales urbanas se generan como consecuencia de la combinación de aguas residuales y otros residuos procedentes de actividades domésticas, comerciales e industriales (Hodaifa et al., 2013). La cantidad generada, así como sus características fisicoquímicas dependen del nivel de vida, el comportamiento y estilo de vida de los habitantes de las regiones donde son generadas. Además, el diseño del sistema de alcantarillado también afecta significativamente a la composición de las aguas (Henze y Comeau, 2008).

1.4.2. Características fisicoquímicas

Las aguas residuales urbanas contienen principalmente compuestos orgánicos, sólidos disueltos y en suspensión, nitrógeno, fósforo, y sales minerales. Además, contienen organismos patógenos, nutrientes y compuestos tóxicos (Saravanane et al., 2014).

Su composición fisicoquímica es muy variable, la concentración de los principales componentes de un agua residual urbana con un bajo contenido en agua residual industrial es la siguiente: DBO_5 (230-560 mg O_2/L), DQO total (500-1200 mg O_2/L), DQO insoluble (300-720 mg O_2/L), DQO soluble (200-480 mg O_2/L), nitrógeno total (30-100 mg/L), nitrógeno amoniacal (20-75 mg/L), fósforo total (6-25 mg/L), ortofosfato (4-15 mg/L), sólidos volátiles en suspensión (200-480 mg/L), sólidos totales en suspensión (250-600 mg/L), ácidos grasos volátiles (10-80 mg/L), Hodaifa et al. (2019).

En cuanto al contenido microbiológico, las aguas residuales contienen diferentes tipos de patógenos, incluyendo virus, bacterias, hongos, gusanos, protozoos, etc. Las bacterias son los microorganismos más abundantes, incluyendo aquellas pertenecientes al género de *Pseudonomas* spp., bacterias fecales como *Escherichia coli* y *Enterococcus* spp. y otras patogénicas para los humanos como *Salmonella* spp. o *Staphylococcus aureus*. Estas bacterias llegan a las aguas residuales principalmente a través de efluentes procedentes de inodoros, lavabos y restos de comida, puesto que la mayoría de ellas se encuentran en los restos fecales humanos (López et al., 2019).

1.4.3. Aplicaciones

Las aguas residuales urbanas tratadas pueden ser reutilizadas en múltiples actividades tales como agricultura, recarga de acuíferos, acuicultura, extinción de incendios, riego de parques y campos de golf y así como prácticamente cualquier actividad que no exija agua potable. La reutilización de dichas aguas depende principalmente de sus características bioquímicas, que determinarán el método y el grado de tratamiento requerido. En este sentido, la reutilización para riego agrícola requiere niveles de tratamiento de menor calidad y la reutilización en actividades domésticas necesita un nivel de tratamiento más elevado (Vigneswaran y Sundaravadivel, 2009).

1.4.3.1. Riego

La reutilización de aguas residuales tratadas para el riego agrícola es la aplicación más antigua y más ampliamente extendida actualmente. Entre las numerosas ventajas, esta aplicación permite la disminución del nivel de purificación de las aguas y, por tanto, el ahorro en los costes del tratamiento de las mismas. Además, numerosos estudios han demostrado que, además de constituir una fuente de agua de bajo coste, tiene numerosos beneficios sobre los cultivos, como el aumento del rendimiento de los mismos y la disminución en el empleo de fertilizantes químicos (Vigneswaran y Sundaravadivel, 2009).

Por otro lado, dentro de las actividades de riego también se incluyen aquellas destinadas al riego de parques públicos, campos de golf, zonas residenciales, etc. Sin embargo, debido a que en estos casos el agua se usa en áreas abiertas al público, existe la posibilidad de que entre en contacto humano, por lo que debe tratarse a un nivel más alto para evitar el riesgo de propagación de enfermedades además de otros problemas como malos olores, insectos, acumulación de nutrientes, etc. (Vigneswaran y Sundaravadivel, 2009). En estos casos se debe de realizar un tratamiento final riguroso de desinfección.

1.4.3.2. Actividades industriales

La reutilización de aguas residuales urbanas tratadas en actividades industriales es la segunda aplicación más extendida después de su uso en riego. Esto se debe a que numerosos

procesos industriales no requieren agua de gran calidad. Además, en muchos casos, las industrias se localizan próximas a las plantas de tratamiento de aguas residuales donde éstas son tratadas. Dependiendo del tipo de industria, el agua recuperada puede emplearse para refrigeración o como agua de alimentación de calderas. En este sentido, su empleo en refrigeración es uno de los usos más extendidos debido a que los requerimientos de calidad no son muy altos (Vigneswaran y Sundaravadivel, 2009).

1.4.4. Problemática ambiental

El crecimiento de la población mundial, la industrialización y el incremento de la urbanización en ciudades han provocado el aumento en la demanda de agua así como en la cantidad de agua residual generada (Meneses et al., 2010).

Además de los grandes volúmenes que son generados, su composición fisicoquímica también supone un gran impacto ambiental debido a la heterogeneidad en su composición, que incluye diversos tipos de compuestos contaminantes tales como metales pesados y una gran variedad de compuestos tóxicos tanto orgánicos como inorgánicos, además de numerosos microorganismos patógenos (Cai y Zhang, 2013; Hodaifa et al., 2019).

El impacto que tiene el vertido de aguas residuales sin tratar en lagos, ríos, embalses, etc., se debe principalmente a tres factores: contaminación por exceso de materia orgánica, contaminación por microorganismos patógenos y eutrofización (Von Sperling, 2015).

En primer lugar, la incorporación de materia orgánica a fuentes naturales de agua provoca el consumo del oxígeno disuelto disponible por parte de bacterias, que proliferan exponencialmente provocando anaerobiosis y como consecuencia, la muerte de peces y animales acuáticos. En segundo lugar, la elevada presencia de microorganismos patógenos en las aguas residuales urbanas supone una gran amenaza tanto para el medio ambiente como para la salud pública debido a la transmisión de enfermedades por parte de estos. Por último, el proceso de eutrofización, causado por un exceso de nutrientes, principalmente nitrógeno y fósforo, da lugar a la proliferación de algas, insectos, malos olores y muerte de peces (Von Sperling, 2015).

1.5. TRATAMIENTO DE AGUAS RESIDUALES

1.5.1. Sistema convencional

El sistema convencional es el método más ampliamente empleado actualmente para el tratamiento de aguas residuales. Dicho sistema se lleva a cabo en estaciones depuradoras de aguas residuales (EDAR) y consta de cuatro etapas: tratamiento preliminar (o pretratamiento), primario, secundario y terciario. A su vez, cada tratamiento está constituido por dos líneas: línea de aguas y de lodos. En algunos casos, se implementan con tratamientos diversos tales como eliminación de metales pesados, ajuste de pH, etc. o desinfección a la salida de la EDAR para cumplir con los requisitos de calidad específicos de la zona de descarga o para mejorar la calidad final del agua tratada. En otros casos específicos, no se requiere el tratamiento preliminar y el proceso puede comenzar directamente con el tratamiento primario (Hodaifa et al., 2019).

El sistema convencional de tratamiento de aguas residuales combina operaciones fisicoquímicas y biológicas para mejorar la calidad del agua mediante la eliminación de la demanda biológica de oxígeno, sólidos en suspensión, nutrientes (nitrato, nitrito, amonio, fosfato...) bacterias coliformes, compuestos tóxicos, etc. (Abdel-Raouf et al., 2012)

1.5.1.1. Pretratamiento

El objetivo de esta primera etapa es la eliminación de materiales sólidos de gran tamaño tales como piedras, trozos de madera, plásticos… que en etapas posteriores pueden obstruir el flujo o dañar los equipos de la planta (Abdel-Raouf et al., 2012).

Este tratamiento consiste en cuatro pasos: separación de arenas, cribado de gruesos, molienda y tratamiento químico. En primer lugar, el objetivo principal de la remoción de arenas es la eliminación de partículas inorgánicas de alta densidad, para ello, se emplea agua o aire a alta velocidad para evitar la sedimentación de sólidos. En segundo lugar, mediante el cribado de gruesos se eliminan los sólidos de mayor tamaño, comúnmente mediante el empleo de rejas que los retienen. El objetivo de la molienda es la disminución del tamaño de las partículas restantes, que una vez reducido su tamaño, se descargan hacia la línea de lodos. Por último, los tratamientos

químicos son eventualmente empleados para mejorar el rendimiento de las etapas posteriores (Hodaifa et al., 2019).

1.5.1.2. Tratamiento primario

Tras la eliminación del material de mayor tamaño, las aguas residuales pasan a tanques de sedimentación con el objetivo de eliminar aquellos sólidos sedimentables en suspensión, tanto orgánicos como inorgánicos, así como materiales voluminosos y pesados que puedan disminuir la eficiencia del proceso en etapas posteriores mediante el bloqueo de equipos tales como tuberías, equipo de bombeo, etc. (Abdel-Raouf et al., 2012).

En esta etapa se pueden emplear tanques de sedimentación circulares o rectangulares. Las aguas residuales pasan a través de los mismos de forma que los sólidos suspendidos, con una densidad mayor que el líquido, se depositan en el fondo. La masa de sólidos que se acumula en el fondo es conocido como lodo primario, que es eliminado a través de una tubería o mediante rascadores mecánicos y bombas. Por otro lado, el material flotante como grasas y aceites, al tener una densidad menor que el líquido circundante, se queda en la superficie de los tanques, donde son retirados (Von Sperling, 2015).

Por último, para mejorar la eficiencia del tratamiento primario, se lleva a cabo una etapa de precipitación química mediante la adición de coagulantes (sulfato de aluminio, cloruro férrico...). Tras la separación del lodo, el líquido resultante se dirige al tratamiento secundario y el lodo se lleva a vertederos o es sometido a digestión anaerobia (Von Sperling, 2015).

1.5.1.3. Tratamiento secundario o biológico

El principal objetivo del tratamiento secundario es la eliminación de la materia orgánica presente en las aguas residuales. Ésta puede encontrarse en forma disuelta, la cual no puede ser eliminada únicamente mediante procesos físicos, o en suspensión, que en gran parte ha sido eliminada en el tratamiento primario pero cuyos sólidos más finos aún permanecen en el líquido (Von Sperling, 2015).

Para ello se lleva a cabo una etapa de tratamiento biológico por microorganismos incluyendo bacterias, protozoos, levaduras y hongos, entre otros. Esta etapa se lleva a cabo en un bioreactor aerobio en el que los microorganismos descomponen la materia orgánica en dióxido de carbono y agua, con formación de material celular (Hodaifa et al., 2019).

El tratamiento secundario se puede llevar a cabo mediante diversos procesos tales como el sistema de lagunas aireadas, filtros percoladores, lodos activos, y digestión anaerobia. Entre ellos, el proceso de lodos activos es la práctica más común. En este sistema, se lleva a cabo la mezcla y agitación del agua residual con lodos biológicos, formados por aglutinación de microorganismos que descomponen la materia orgánica (Von Sperling, 2015). Por último, los materiales más densos del agua se depositan en el fondo del tanque de sedimentación (previa corrección del pH), formando el lodo secundario, y el agua residual se conduce a tratamiento terciario o a su vertido a cauce público (Hodaifa et al., 2019). En este último caso el agua debería ser siempre desinfectada.

1.5.1.4. Tratamiento terciario

Tiene como objetivo la eliminación de compuestos orgánicos, nutrientes, sólidos en suspensión, microorganismos y otros contaminantes que no han sido eliminados en el tratamiento secundario y que es necesario separar para obtener un agua que cumpla con la normativa para ser reutilizada y vertida en zonas sensibles. Éste puede realizarse mediante procesos físicos, químicos, biológicos independientes o combinados (Abdel-Raouf et al., 2012).

Los tratamientos terciarios que se suelen emplear se basan en técnicas complejas tales como tratamientos fisicoquímicos como la coagulación-floculación, la tecnología de membranas o sistemas extensivos con materiales filtrantes tales como arena, carbón activo y zeolita. La elección del sistema empleado depende de diversos factores, siendo la composición y la cantidad de aguas que tratar el más relevante y que vendrá determinado por el origen del agua residual. Además, el agua tratada debe cumplir con la normativa de acuerdo con el uso que se le vaya a dar: riego, recarga de acuíferos, etc. La tecnología disponible y la generación de subproductos y su gestión son otros factores a tener en cuenta (Salgot et al., 2018).

1.5.2. Sistemas no convencionales

Los sistemas no convencionales de tratamiento de aguas residuales han sido ampliamente desarrollados en los últimos años como alternativa a los sistemas tradicionales, que presentan ciertos inconvenientes tales como el requerimiento de grandes superficies, emisiones procedentes de grandes reactores abiertos, gran producción de lodos y alto consumo de energía. Es por ello que en los últimos años se han desarrollado nuevos métodos para superar los inconvenientes que presentan los sistemas convencionales (Sikosana et al., 2019).

1.5.2.1. Floculación

Las aguas residuales se caracterizan por contener sólidos en suspensión y disueltos de pequeño tamaño, partículas orgánicas e inorgánicas, metales, etc. El pequeño tamaño de estas partículas y su carga superficial dificulta el proceso de agregar estas partículas y convertirlas en una masa más pesada para su sedimentación y eliminación. En este sentido, la floculación ha demostrado ser un método muy eficiente para el tratamiento de aguas residuales mediante procesos de separación sólido-líquido (Lee et al., 2014).

La floculación directa consiste en la neutralización de la carga de partículas coloidales para la posterior formación de flóculos de gran tamaño. Para ello se emplean floculantes, polímeros catiónicos de alto peso molecular y densidad de carga media cuya función es neutralizar la carga negativa de las partículas coloidales y unir las partículas desestabilizadas para formar flóculos. Los floculantes pueden agruparse en dos categorías: floculantes químicos (polímeros orgánicos sintéticos) y floculantes naturales (quitosano, celulosa, alginato de sodio, taninos…), Lee et al. (2014).

Este sistema de tratamiento presenta diversas ventajas, como la generación de una menor cantidad de lodos debido a la formación de enlaces más fuertes entre los flóculos, que dan lugar a lodos más densos y comprimidos. Además, al emplearse polímeros de naturaleza orgánica, la mayoría de los lodos generados pueden ser desechados sin requerir un tratamiento previo, reduciendo así los costes del proceso. La floculación directa ha demostrado ser efectiva para el tratamiento de diversos tipos de aguas residuales tales como aguas residuales de almazara, de

acuicultura, de la industria del papel y textil, consiguiendo un alto rendimiento en la eliminación de la turbidez, sólidos totales, DQO y color de las mismas (Lee et al., 2014).

1.5.2.2. Fotólisis ultravioleta

La fotólisis es una operación fotoquímica en la que compuestos orgánicos son parcialmente descompuestos debido a la absorción de radiación de alta energía. Las tecnologías fotoquímicas se han desarrollado notablemente durante los últimos años como sistema de eliminación de contaminantes en las aguas residuales. Éstas son sencillas y limpias, rentables en numerosas aplicaciones y a menudo proporcionan la doble ventaja de eliminar contaminantes presentes en las aguas residuales y desinfectar (Stefan, 2004).

Los procesos de oxidación avanzada con luz ultravioleta (UV) se basan en la generación de potentes especies oxidantes, tales como el radical hidroxilo, mediante fotólisis directa de peróxido de hidrógeno, o mediante procesos foto inducidos tales como Foto-fenton o fotocatálisis. Por otro lado, en la fotólisis UV directa, los contaminantes deben absorber la radiación y degradarse a partir de su estado de excitación (Stefan, 2004).

Actualmente, existe un gran interés en la aplicación de luz UV para el tratamiento de contaminantes presentes en las aguas residuales debido principalmente al efecto tóxico y carcinogénico que pueden tener los mismos. Además, los requerimientos respecto a los niveles admitidos de determinados contaminantes en medios acuáticos son cada vez más restrictivos por parte de las agencias reguladoras (Stefan, 2004).

La mayoría de los compuestos que absorben luz UV contienen dobles enlaces o dobles enlaces conjugados, incluyendo carbón, nitrógeno, o átomos de oxígeno y se caracterizan por tener electrones deslocalizados. Estos sistemas se denominan cromóforos. Los contaminantes ambientales que contienen estructuras cromóforas incluyen alquenos, compuestos aromáticos y heterocíclicos, aldehídos, cetonas, acidos carboxílicos, nitroderivados… (Stefan, 2004).

En fotólisis se pueden emplear diferentes tipos de lámparas UV (mercurio, xenón, LEDs, etc.) con diferentes rangos de emisión y potencia. En general, las lámparas de mercurio de baja y

media intensidad son las más empleadas actualmente en el tratamiento de aguas residuales (García y Hodaifa, 2017).

En las lámparas de mercurio de baja intensidad, más del 80% de las emisiones ocurren a 254 nm y la fracción restante a 185 nm, esto les aporta una gran eficiencia en la descomposición de contaminantes puesto que ambas radiaciones se encuentran en el espectro de absorción de la mayoría de los contaminantes. Este tipo de lámparas trabaja a una baja potencia (entre 5-80 W), desde temperatura ambiente hasta 40ºC. Se emplean mayormente para la desinfección de agua potable, así como en la industria farmacéutica y la alimentaria. Por otro lado, las lámparas de mercurio de media intensidad pueden tener una potencia desde 100 a 1000 W, con un perfil de emisión en el rango de 200-700 nm (García y Hodaifa, 2017). Su empleo es muy común en numerosas aplicaciones fotoquímicas, sobre todo aquellas relacionadas con el tratamiento de contaminantes ambientales (Stefan, 2004). Por último, las lámparas de alta intensidad pueden tener una potencia desde 150 a 1000 W y operan a altas temperaturas. Estas lámparas de mercurio son las más potentes y de menor tamaño (García y Hodaifa, 2017).

1.5.2.3. Cultivo de microalgas

El cultivo de microalgas empleando aguas residuales como medio de cultivo es una tecnología novedosa con la que se consigue llevar a cabo el tratamiento de las aguas residuales a la vez que se genera biomasa microalgal con un alto valor añadido (Sánchez et al., 1996; Mata et al., 2010).

Esta aplicación es posible gracias a la habilidad de las microalgas de asimilar los nutrientes presentes en las aguas residuales (macro, micronutrientes y elementos traza) para su crecimiento. Numerosas especies pertenecientes a diversos géneros como *Chlorella, Scenedesmus* o *Neochloris,* entre otros muchos, han demostrado su capacidad de crecimiento en condiciones extremas eliminando diversos nutrientes, contaminantes, metales pesados, compuestos nitrogenados, etc. Este hecho permite el tratamiento de diferentes tipos de aguas residuales tales como aguas residuales de acuicultura, domésticas, urbanas e industriales (Wang et al., 2016).

Esta tecnología presenta numerosos beneficios: es sostenible y respetuosa con el medio ambiente, durante la fotosíntesis las microalgas producen oxígeno y consumen dióxido de carbono y se lleva a cabo la biorremediación de compuestos tanto orgánicos como inorgánicos. En este sentido, las microalgas tienen una gran capacidad de eliminar nitrógeno y fósforo de las aguas residuales, lo cual es de gran importancia para evitar serios problemas ambientales tales como la eutrofización. Además, es una tecnología que no requiere costes elevados y con la que se genera una biomasa de alto valor añadido que puede ser empleada en diversas aplicaciones tales como generación de biocombustibles, alimentación humana y animal e industria farmacéutica. (Pittman et al., 2011).

1.6. MICROALGAS

Las microalgas son organismos unicelulares que se encuentran en una gran diversidad de hábitats mayormente acuáticos, aunque también pueden encontrarse en suelos de todo tipo, organizadas en colonias, en simbiosis o como células independientes. Las microalgas son microorganismos fototróficos, por lo que son capaces de realizar la fotosíntesis (Tomaselli, 2004). El gran número de especies que existe se subdividen en diez grupos taxonómicos que incluyen las algas verdes (Chlorophyceae), diatomeas (Bacillar-iophyceae), amarillo-verdes (Xanthophyceae), algas doradas (Chrysophyceae), algas rojas (Rhodophyceae), algas pardas (Phaeophyceae), dinoflageladas (Dinophyceae), Prasinophyceae y Eustigmatophyceae. Las algas verde-azules (Cyanophyceae) fueron originalmente agrupadas con las algas eucariotas, sin embargo, posteriormente se descubrió que pertenecen al dominio de las bacterias, de ahí su nombre actual común cianobacterias (Williams y Laurens, 2010).

El interés por estos microorganismos fototróficos reside en las numerosas aplicaciones a las que se pueden destinar: producción de biomasa para alimentación, productos químicos, compuestos de alto valor añadido… todo ello con la doble ventaja de la utilización de energía solar como fuente de energía (Tomaselli, 2004).

1.6.1. Morfología, organización y composición bioquímica

Las microalgas pueden presentar diversos tipos de morfología y organización celular: microalgas unicelulares, organizadas en colonias, filamentosas y flageladas. A su vez, éstas pueden o no presentar movilidad, debiéndose la motilidad a la presencia de flagelos. Por otro lado, en microalgas móviles, las células flageladas pueden agregarse para formas colonias móviles (es el caso de *Volvox*) o no móviles (tal como *Gloeocystis*). A su vez, las células no móviles se pueden organizar con un número fijo de células en la colonia (*Scenedesmus*) o con un número variable (*Pediastrum*). Por otro lado, las microalgas que presentan células filamentosas ya sean no ramificadas o ramificadas, no suelen presentar movilidad (Tomaselli, 2004).

En cuanto a la organización celular, las microalgas poseen un núcleo delimitado por una membrana, que contiene la mayor parte del genoma distribuido en un conjunto de cromosomas y

el nucléolo. Su citoplasma se encuentra dividido en compartimentos y orgánulos unidos a la membrana: aparato de Golgi, mitocondrias, retículo endoplasmático, vacuolas, centriolos y plastidios, dedicados a funciones específicas. Por otra parte, aquellas microalgas que presentan una organización celular multinucleada generalmente tienen un citoplasma periférico donde se encuentran los núcleos y cloroplastos (Tomaselli, 2004).

En cuanto a la composición bioquímica de las microalgas hay que considerar cuatro grupos principales de moléculas: carbohidratos, proteínas, ácidos nucleicos y lípidos.

i. Carbohidratos.

Su función es tanto estructural como metabólica y son los primeros productos de la fotosíntesis, sirviendo como precursores para la síntesis de numerosas moléculas. Diferentes clases de microalgas producen diferentes tipos específicos de polisacáridos. Por ejemplo, las algas verdes producen almidón como reserva de energía, al igual que ocurre en las plantas superiores. El alga verde *Tetraselmis suecica* acumula entre el 11 y 47% de su peso seco en forma de este carbohidrato. Por otro lado, un polisacárido muy común en un gran número de especies de microalgas es la crisolaminarina, un polímero lineal de beta (1,3) y beta (1,6) vinculado a unidades de D-glucosa; éste a menudo se acumula en unas estructuras de alta actividad de asimilación de carbono conocidas como pirenoides que se encuentran en los cloroplastos (Williams y Laurens, 2010).

ii. Proteínas.

Las proteínas, al igual que los carbohidratos, desempeñan funciones tanto estructurales como metabólicas. Dentro de este grupo bioquímico se encuentran las enzimas, principales catalizadores en el metabolismo celular que además desempeñan funciones estructurales proporcionando el andamio sobre el cual las moléculas de clorofila se ensamblan en los complejos de recolección de luz del cloroplasto. Éstas además se pueden encontrar insertadas en las membranas lipídicas, así como en la pared celular de numerosas especies de microalgas como ocurre con *Clamydomonas reinhardtii*, cuya pared celular consiste principalmente en glicoproteínas ricas en hidroxiprolina (Williams y Laurens, 2010).

iii. Ácidos nucleicos.

Los ácidos nucleicos asociados a proteínas proporcionan la base para el crecimiento y división de las microalgas. Éstos comprenden una pequeña fracción de la biomasa celular, concentrando la mayor parte del fosfato de la célula y el segundo sitio más importante de nitrógeno (Williams y Laurens, 2010).

iv. Lípidos.

Los lípidos desempeñan dos tipos de funciones: reserva energética (esencialmente son triglicéridos de ácidos grasos simples) y componentes estructurales de las células. Los segundos se encuentran localizados principalmente en las membranas celulares, constituidas de fosfolípidos y glicolípidos y con un papel de gran importancia en la capacidad de las microalgas para adaptarse a nuevos ambientes y condiciones, puesto que son capaces de llevar a cabo la síntesis *de novo* y reciclaje de ácidos grasos para el mantenimiento de las propiedades de las membranas (Williams y Laurens, 2010).

En cuanto a la composición en ácidos grasos de los lípidos de las microalgas, estas se caracterizan por tener una elevada proporción de ácidos grasos insaturados y poliinsaturados, encontrándose la mayoría de ellos en las membranas celulares, en las que juegan un papel crucial en el mantenimiento de la fluidez de la membrana bajo distintas condiciones (Williams y Laurens, 2010).

1.6.2. Metabolismo

Por lo general, las microalgas son organismos fotoautótrofos, aunque son capaces de adaptar su metabolismo a las condiciones medioambientales en las que se hallan creciendo. En función de la fuente de energía y de carbono, las microalgas pueden presentar diferentes tipos de metabolismo:

1.6.2.1. Cultivos autotróficos

Este tipo de metabolismo se caracteriza por el empleo de luz como única fuente de energía mediante su conversión, a través de la fotosíntesis, en energía química y por el empleo de carbono inorgánico, como el dióxido de carbono, como fuente de carbono (Martínez-Sancho, 1980). Este es el método de cultivo más ampliamente utilizado para el crecimiento de microalgas (Chen et al., 2011; Chojnacka y Marquez-Rocha, 2004).

1.6.2.2. Cultivos heterotróficos

El metabolismo heterótrofo se caracteriza por la utilización de compuestos orgánicos como fuente de carbono y energía, por lo que este tipo de metabolismo no requiere de la presencia de luz (Sánchez, 1986; Chojnacka y Marquez-Rocha, 2004).

1.6.2.3. Cultivos mixotróficos

Este tipo de metabolismo se produce cuando las microalgas realizan la fotosíntesis para la obtención de energía y emplean compuestos orgánicos y carbón inorgánico (CO_2) como fuente de carbono para su crecimiento. Esto significa que las microalgas son capaces de vivir tanto en condiciones fototróficas como heterotróficas (Martínez-Sancho, 1980; Chen et al., 2011).

1.6.2.4. Cultivos fotoheterotróficos

Se trata de un tipo metabolismo en el que se requiere la presencia de luz como fuente de energía para la utilización de compuestos orgánicos como fuente de carbono. Por tanto, en este tipo de cultivos se requieren compuestos orgánicos y luz al mismo tiempo (Sánchez, 1986; Chen et al., 2011).

1.6.3. Aplicaciones

1.6.3.1. Alimentación animal

El empleo de microalgas para alimentación animal es una aplicación muy extendida actualmente. Numerosos estudios han demostrado su idoneidad como suplemento o sustituto de

fuentes tradicionales de proteínas como la harina de soja, la harina de pescado o el salvado de arroz (Becker, 2007).

Los criterios nutricionales que deben cumplir las microalgas para su empleo en alimentación animal y acuicultura son los siguientes: no deben ser tóxicas, deben tener un tamaño aceptable para la ingestión y su pared celular ha de ser digerible. Además, respecto a su composición lipídica, la calidad de los mismos es primordial frente a la cantidad, ya que en función de la cantidad de ácidos grasos esenciales presentes en las microalgas, las larvas de peces pueden ser saludables o crecer con formaciones incorrectas (Mata et al., 2010)

Su uso más ampliamente extendido es en el sector de la acuicultura, es tanto como alimento tanto de animales acuáticos de agua dulce como de agua salada. También son empleadas como fuente de alimentación para el cultivo de diversos tipos de zooplancton, que a su vez son empleados como alimento de crustáceos y peces de piscifactoría (Mata et al., 2010).

De entre las especies más empleadas en este sector hay que mencionar *Isochrysis galbana* y *Tetraselmis suecica,* ampliamente utilizadas como alimento de bivalvos. Por otro lado, *Scenedesmus* se emplea como alimento de *Artemia* y por último, *Chlorella* es empleada para el cultivo del rotífero *Brachionus plicatilis* (Mata et al., 2010).

Se estima que en torno al 30% de la producción algal a nivel mundial es destinada a alimentación animal (Becker, 2007).

1.6.3.2. Alimentación humana

El empleo de microalgas en alimentación humana se debe a diversos factores tales como su contenido en proteínas, cuyo valor nutricional es mucho más elevado en comparación con otras fuentes vegetales tales como el trigo o el arroz. Además, las microalgas son una importante fuente de compuestos bioactivos tales como ácidos grasos, carotenoides, β−caroteno, astaxantina, o luteína. Todos ellos caracterizados por su elevado valor nutricional y de vital importancia en el tratamiento y prevención de diversas enfermedades humanas (Suganya et al., 2016).

Chlorella, Dunaliella y Spirulina son géneros predominan en este sector. En primer lugar, *Chlorella* se destaca por sus numerosas propiedades para la salud humana, así como su eficacia en el tratamiento de úlceras gástricas, aterosclerosis e hipercolesterolemia entre otras muchas patologías. Además, la ingesta de extractos procedentes de *Chlorella* ha demostrado tener numerosos beneficios para la salud tales como el aumento de la concentración de hemoglobina y la disminución de los niveles de azúcar en sangre. En segundo lugar, *Dunaliella* sp.*, y* especialmente *Dunaliella salina*, ha suscitado un gran interés en este sector debido a su elevado contenido en lípidos y proteínas, así como glicerol y β–caroteno, compuesto que puede llegar a alcanzar hasta un 14% del peso seco de dicha especie. Por último, *Spirulina* sp. constituye una importante fuente de proteínas, ácidos grasos esenciales (ácido linoleico), carotenoides y diversos compuestos antioxidantes. Su consumo está asociado a la disminución de la hipertensión, de la hiperlipidemia (exceso de grasa en la sangre) y de la insuficiencia renal, entre otros muchos beneficios (Suganya et al., 2016).

1.6.3.3. Producción de biodiesel

El biodiesel es un biocombustible sintetizado a partir de biomasa renovable capaz de sustituir al diésel derivado del petróleo como combustible. Éste se produce mediante transesterificación, reacción química ocurrida entre triglicéridos y un alcohol (comúnmente metanol, etanol, propanol o butanol) y que da lugar a ésteres (biodiesel) y glicerol (subproducto). Además, esta reacción requiere de la presencia de un catalizador para reducir el tiempo de reacción. Éstos pueden ser homogéneos o heterogéneos, básicos o ácidos, siendo el NaOH y el KOH los más comúnmente empleados en procesos industriales (Mata et al., 2010).

Actualmente el biodiesel comercial se produce a partir de diversos tipos de aceites vegetales (palma, soja, colza maíz, palma, coco…). Sin embargo, esta práctica es controvertida debido a la escasez de tierras disponibles para los cultivos destinados a alimentación humana. Es por ello que las microalgas han despertado un gran interés como fuente alternativa de biomasa para la producción de este biocombustible. Entre las numerosas ventajas que éstas presentan, cabe destacar su rápido crecimiento y su capacidad de convertir energía solar en energía química

mediante fotosíntesis, fijando CO_2. Además, bajo unas condiciones de cultivo adecuadas, algunas especies de microalgas pueden acumular hasta un 50-75% de lípidos en relación a su peso seco, caracterizándose dichos lípidos por tener un perfil de ácidos grasos adecuado para la síntesis de biodiesel. Por último, éstas pueden ser cultivadas en tierras no fértiles sin afectar así a otros cultivos destinados a la alimentación humana (Chen et al., 2011).

1.6.3.4. Biomitigación de CO_2

Las grandes emisiones de CO_2 por parte de industrias y centrales eléctricas suponen un serio problema ambiental actualmente. En este sentido, dos estrategias son empleadas para la mitigación de este gas de efecto invernadero. Por un lado, se encuentran las tecnologías basadas en reacciones químicas, que suponen un elevado consumo de energía, altos costes y serios problemas de generación de residuos derivados del uso de materiales o compuestos absorbentes (Mata et al., 2010).

Por otro lado, la biomitigación mediante procesos biológicos ha despertado un gran interés debido a la posibilidad de generar biomasa a la vez que se lleva a cabo la fijación de CO_2. En este sentido, las microalgas juegan un papel muy relevante gracias a su capacidad de capturar CO_2 mediante la fotosíntesis, pudiendo emplearse para captar las emisiones de las centrales eléctricas y procesos industriales. En este sentido, es de vital importancia la selección de la especie adecuada. No solo debe ser tolerante a altas concentraciones de CO_2, también deben tolerar altos niveles de SO_x y NO_x, presentes en los gases de combustión de las centrales eléctricas. Además, debe presentar una alta tasa de crecimiento, generación de subproductos de alto valor añadido, facilidad de recolección y una elevada tolerancia a la temperatura del agua para minimizar costes de refrigeración de los gases. Diversas cepas pertenecientes al género *Chlorella*, *Scenedesmus* o *Botryococcus* han demostrado su viabilidad para esta aplicación (Órpez et al., 2009; Mata et al., 2010).

1.6.4. Sistemas de cultivo

1.6.4.1. Sistemas cerrados

En los sistemas de cultivo cerrados, conocidos como fotobiorreactores, la luz debe pasar a través de la pared transparente del reactor para alcanzar el cultivo. Con los sistemas cerrados se abordan algunos de los problemas asociados a los sistemas abiertos tales como la evaporación del agua así como el crecimiento de microorganismos y agentes patógenos no deseados (Razzak et al., 2013). Además, el empleo de fotobiorreactores permite el control de todos los parámetros de cultivo (Faried et al., 2017).

i. Columnas de burbujeos

Las columnas verticales suelen ser cilindros de hasta 0,2 metros de radio y altura máxima de 4 metros. Estas columnas presentan radios pequeños para aumentar la relación superficie-volumen. Por otro lado, la restricción de altura se asocia con las limitaciones de transferencia de gas y la fuerza de los materiales transparentes utilizados para su construcción (Wang et al., 2012). En este sentido, el polietileno y el vidrio son los materiales más comúnmente empleados (Carvalho y Meireles, 2006).

Dentro de este tipo de fotobiorreactores se pueden encontrar dos configuraciones: columna de burbujeo y reactor con puente aéreo (comúnmente conocido como reactor *airlift*), ambas con estructura similar pero diferentes componentes. En el primer caso, las columnas de burbujeo están constituidas por un inyector de aire en la zona inferior y un régimen en la zona superior que permite la separación gas/líquido. La mezcla del cultivo se logra por la turbulencia creada por las burbujas de aire enriquecido con CO_2 que se desplazan hacia la zona superior del reactor (Chew et al., 2018). En segundo lugar, los reactores *airlift* se componen de dos partes que se encuentran interconectadas: la zona ascendente, por la que se inyecta el aire que provoca el movimiento del líquido hacia la zona superior del mismo, y la zona descendente, que no recibe aire y en la que cae el líquido tras haber sido desgasificado en la zona superior del reactor (Singh y Sharma, 2012). A su vez, los reactores *airlift* se pueden encontrar en diferentes configuraciones incluyendo en su estructura una pequeña columna interna transparente con un inyector de aire en

la parte inferior. Una variante de esta configuración es el reactor *airlift* de bucle externo, con una columna de circulación externa. Por último, también pueden presentar una placa plana que divide la columna en dos partes, una para la inyección del aire y la otra para la recepción del líquido (Wang et al., 2012).

ii. Reactores tubulares

La configuración del fotobiorreactor tubular incluye una serie de tubos transparentes que se pueden disponer de forma vertical, horizontal o en espiral. El diámetro de dichos tubos no debe sobrepasar los 0,1 metros para garantizar así una alta productividad de biomasa (Wang et al., 2012).

Un fotobiorreactor tubular se compone de los siguientes elementos: matriz solar para el crecimiento de las microalgas, unidad de recolección para separar las microalgas de la suspensión, columna de desgasificación para el intercambio de gases, refrigeración e introducción del medio fresco y bomba de circulación (Wang et al., 2012).

Los reactores tubulares horizontales (RTH) consisten en una serie de tubos en paralelo dispuestos horizontalmente con un intercambiador de gas a través del cual se inyecta CO_2. En este tipo de reactores, el medio de cultivo es bombeado a través de los tubos permitiendo mantener un elevado régimen de flujo turbulento que previene la sedimentación de las microalgas (Chew et al., 2018). La principal ventaja que presentan los RTH es la alta eficiencia de conversión de luz debido a la posibilidad de orientar el reactor hacia la luz solar, eliminando así la necesidad de aplicar iluminación artificial (Singh y Sharma, 2012). Sin embargo, este hecho provoca la generación de elevadas cantidades de calor, lo cual requiere de sistemas de control de temperatura (Wang et al., 2012). Además, este sistema requiere de una gran área de terreno debido a la elevada superficie que presentan los RTH (Chew et al., 2018).

Por otro lado, otro posible diseño son los reactores tubulares helicoidales, los cuales consisten en una serie de tubos flexibles de pequeño diámetro (entre 2,5 y 5 cm) dispuestos en forma de espiral. Los materiales más ampliamente empleados para su construcción son el polietileno y el PVC. En esta configuración, los tubos se disponen alrededor de un soporte

cilíndrico y se conectan a una bomba que permite el flujo constante de la suspensión (Tredici, 2004). Este tipo de reactores ha sido demostrado ser apto para cultivos al aire libre empleando luz solar, lo cual permite la reducción de los costes de producción (Razzak et al., 2013).

Por último, otra posible configuración dentro de los reactores tubulares son los conocidos como reactores *alpha-shaped*. Estos se constituyen por una serie de tubos transparentes, comúnmente hechos de PVC y equipados con una bomba que promueve la trayectoria ascendente/descendente del cultivo, así como varios inyectores de CO_2 a lo largo de los tubos (Carvalho y Meireles, 2006).

iii. Fotobiorreactores de pared

Estos fotobiorreactores se componen de una serie de paneles estrechos diseñados para lograr una elevada relación área/volumen para una máxima eficiencia en el uso de la luz (Carvalho y Meireles, 2006). Se caracterizan por el pequeño espesor de las placas que permite una mejor distribución y difusión de la luz. En general, cuanto más corta es la trayectoria de la luz y mayor es la superficie de iluminación, mayor es la eficiencia fotosintética y por lo tanto la densidad celular y la productividad de biomasa (Wang et al., 2012).

Este sistema se puede emplear tanto en exteriores como interiores, con luz artificial o natural y se pueden clasificar en dos categorías principales en función del mecanismo empleado para la mezcla de cultivo. Por un lado, están aquellos compuestos por una bomba que genera un flujo de líquido y como consecuencia una turbulencia que da lugar a la mezcla, y en segundo lugar están aquellos en los que la mezcla se lleva a cabo mediante la inyección de aire comprimido (Ugwu et al., 2008; Wang et al., 2012).

1.6.4.2. Sistemas abiertos

Los estanques abiertos han sido ampliamente utilizados para el cultivo de microalgas a gran escala (Tredici, 2004). Este tipo de sistema presenta diversas ventajas como la disminución en los costes de construcción, así como una mayor facilidad de operación. Además, permite una mayor capacidad de producción respecto a otros sistemas de cultivo (Singh y Sharma, 2012). Sin

embargo, la sensibilidad de estos sistemas a las condiciones meteorológicas, los problemas asociados a la contaminación con otros microorganismos, las pérdidas de evaporación, así como la difusión de CO_2 a la atmósfera son algunas de las limitaciones que presentan los sistemas abiertos (Tredici, 2004; Ugwu et al., 2008).

Dentro de los estanques abiertos se incluyen tanto lagos y lagunas naturales como estanques artificiales en diferentes configuraciones tales como estanques circulares, inclinados y 'raceway'. Éstos difieren en tamaño, conformación, material, sistema de agitación e inclinación (Singh y Sharma, 2012).

i. Estanques naturales

Este sistema constituye la alternativa más simple y económica para el cultivo de microalgas a gran escala. Para su construcción se emplean comúnmente estanques naturales de agua con menos de medio metro de profundidad y sin sistema de agitación, lo cual exige de unas condiciones climáticas adecuadas y suficientes nutrientes para el crecimiento de las microalgas (Razzak et al., 2013).

El principal inconveniente de los estanques naturales sin agitación es la limitación en el crecimiento de las microalgas provocada por su exposición a condiciones ambientales adversas, así como el crecimiento simultáneo de protozoos, bacterias y virus (Razzak et al., 2013).

ii. Estanques circulares

Estos sistemas se construyen de hormigón y se caracterizan por tener una profundidad de aproximadamente 25-30 centímetros y un diámetro que puede alcanzar hasta los 45 metros. Su tamaño no puede exceder los 10.000 m^2 para garantizar la efectividad del sistema de agitación, constituido por un brazo central giratorio que permite la mezcla homogénea del cultivo, así como la exposición de las microalgas a la luz solar (Faried et al., 2017).

Este sistema no se emplea a escala comercial debido a los elevados costes de construcción y operación, así como la elevada energía que requieren para el mezclado (Faried et al., 2017).

iii. Fotobiorreactor '*Raceway'*

Este constituye el sistema de cultivo abierto más popular que se emplea actualmente para el cultivo de numerosas especies de microalgas con fines comerciales. Se caracterizan por tener una profundidad de entre 15-25 centímetros y pueden ser construidos como un único canal o como un conjunto de canales (Razzak et al., 2013). Estos fotobiorreactores generalmente se construyen con cemento y presentan un sistema de agitación con palas giratorias que permite el mezclado del agua a alta velocidad para evitar la deposición y agregación de las microalgas (Singh y Sharma, 2012). Además, este sistema de agitación permite que todas las células reciban luz solar de forma homogénea, así como el intercambio de CO_2 con la atmósfera (Chew et al., 2018).

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2. OBJETIVOS/OBJECTIVES

España es actualmente el mayor productor de aceites de oliva a nivel mundial, con una media de producción en las diez últimas campañas (2009/10 – 2018/19) de $1{,}34\times10^6$ toneladas al año. Como consecuencia, esta elevada producción ha desencadenado una serie de desafíos respecto a los residuos generados durante el proceso de extracción del aceite de oliva y respecto a la autentificación de los aceites de oliva producidos.

Por un lado, la industria oleícola genera enormes volúmenes de aguas residuales procedentes de las almazaras, en las que se incluyen las aguas de lavado de aceite y las aguas de lavado de aceitunas. Éstas se caracterizan por tener una elevada carga orgánica, así como una alta concentración de sólidos en suspensión, restos de aceite, etc. Esto da lugar a que dichas aguas residuales tengan un gran impacto ambiental y que su tratamiento sea altamente complicado. Hoy en día, la práctica más extendida es su gestión mediante la acumulación las aguas residuales en grandes balsas para su evaporación durante los meses de verano. Sin embargo, esta gestión da lugar a numerosos problemas tales como la no eliminación total de estas aguas residuales produciendo concentrados de las mismas, contaminación de aguas subterráneas, malos olores, etc. Es por ello que la búsqueda de nuevos tratamientos efectivos es de gran importancia para así disminuir el impacto ambiental de las mismas y poder llevar a cabo su reutilización.

Por otro lado, el consumo de aceite de oliva se ha incrementado notablemente a nivel mundial debido a sus numerosas propiedades nutricionales que se traducen en importantes beneficios para la salud humana. Estos atributos se deben principalmente a su composición química. Sin embargo, ésta puede verse afectada por procesos de oxidación, procesamiento térmico o malas prácticas. Además, otro factor de gran importancia es el correcto etiquetado del aceite de oliva, así como evitar su adulteración con otros aceites de menor calidad. La búsqueda de nuevas técnicas rápidas, precisas y económicas que garanticen la calidad del aceite de oliva puede suponer un gran avance para la industria oleícola a nivel mundial.

A continuación, se describen los objetivos específicos de la presente Tesis Doctoral divididos en los capítulos en los que se abarcan:

2.1. Proceso integrado para el tratamiento de aguas residuales de almazara y su revalorización mediante la generación de biomasa microalgal de alto valor añadido.

1. Caracterización fisicoquímica de las aguas residuales industriales procedentes de la industria del aceite.
2. Diseño de un bioproceso compuesto por un tratamiento fisicoquímico primario y un tratamiento biológico secundario para el tratamiento de las aguas residuales de almazara y su aprovechamiento como medio de cultivo microalgal.
3. Combinación de las siguientes operaciones fisicoquímicas como tratamiento primario:
    i. Floculación-sedimentación.
    ii. Fotólisis artificial mediante la aplicación de luz ultravioleta.
    iii. Microfiltración con membrana.
4. Determinación de la eficacia de eliminación de contaminantes del tratamiento primario global, así como de cada una de las operaciones fisicoquímicas, mediante la caracterización del agua final y el cálculo de los porcentajes de eliminación.
5. Empleo de diferentes concentraciones de agua residual tras el tratamiento primario como medio de cultivo de la microalga *Chlorella pyrenoidosa.*
6. Estudio cinético del crecimiento de *Chlorella pyrenoidosa* en las diferentes concentraciones de agua residual mediante la determinación de la velocidad específica máxima de crecimiento y la productividad de la biomasa.
7. Determinación de la composición bioquímica de la biomasa microalgal, en términos de proteínas, lípidos y carbohidratos, obtenida al final de cada cultivo.
8. Estudio de la concentración de los principales contaminantes en las aguas residuales a lo largo del cultivo de *C. pyrenoidosa.*
9. Cálculo de la eficacia del cultivo microalgal como agente de bioremediación mediante la caracterización de las aguas finales y el cálculo de los porcentajes de eliminación.

2.2. Combinación de operaciones fisicoquímicas y cultivo de microalgas como un nuevo bioproceso para el tratamiento de las aguas residuales de almazara.

1. Caracterización fisicoquímica de las aguas residuales industriales procedentes de la industria del aceite de oliva.
2. Diseño de un bioproceso compuesto por un tratamiento fisicoquímico primario y un tratamiento biológico secundario para el tratamiento de las aguas residuales de almazara y su aprovechamiento como medio de cultivo microalgal.
3. Combinación de las siguientes operaciones fisicoquímicas como tratamiento primario:
   i. Floculación-sedimentación.
   ii. Microfiltración con membrana.
4. Determinación de la eficacia de eliminación de contaminantes del tratamiento primario global, así como de cada una de las operaciones fisicoquímicas mediante la caracterización del agua final obtenida y el cálculo de los porcentajes de eliminación.
5. Empleo de diferentes concentraciones de agua residual tras el tratamiento primario como medio de cultivo de la microalga *Scenedesmus obliquus*.
6. Estudio cinético del crecimiento de *S. obliquus* en las diferentes concentraciones de agua residual mediante la determinación de la velocidad específica máxima de crecimiento y la productividad de biomasa.
7. Determinación de la concentración de biomasa final y su composición bioquímica, en términos de proteínas, lípidos y carbohidratos, obtenida al final de cada cultivo.
8. Estudio de la concentración de los principales contaminantes presentes en las aguas residuales a lo largo del cultivo de *S. obliquus*.
9. Cálculo de la eficacia del cultivo microalgal como agente de bioremediación de las aguas residuales de almazara mediante la caracterización de las aguas finales y el cálculo de los porcentajes de eliminación.
10. Establecimiento de las mejores condiciones de operación en base al crecimiento microalgal, la producción de biodiesel y la eliminación de contaminantes.

2.3. Cultivo de *Scenedesmus obliquus* en mezclas de aguas residuales urbanas y aguas de almazara para la producción de biomasa microalgal y el tratamiento de las aguas residuales.

1. Caracterización fisicoquímica del agua residual urbana procedente del tratamiento terciario de una estación depuradora de aguas residuales urbanas y de agua residual de almazara.
2. Estudio del empleo de agua residual urbana, así como mezclas de agua residual urbana con agua residual de almazara (esta última pretratada mediante floculación-sedimentación y fotólisis UV) como medio de cultivo de *Scenedesmus obliquus*.
3. Estudio cinético del crecimiento de *S. obliquus* en los medios de cultivo descritos en el punto anterior mediante la determinación de la velocidad específica máxima de crecimiento y la productividad de biomasa.
4. Determinación de la composición bioquímica de la biomasa microalgal, en términos de proteínas, lípidos y carbohidratos, obtenida al final de cada cultivo.
5. Estudio de la concentración de los principales contaminantes en los diferentes medios a lo largo del cultivo de *S. obliquus*.
6. Cálculo de la eficacia del cultivo microalgal como agente de bioremediación mediante la caracterización de las aguas residuales finales y el cálculo de los porcentajes de eliminación.

2.4. Determinación de la estabilidad por oxidación térmica y de los parámetros cinéticos de diferentes variedades de aceite de oliva virgen extra.

1. Estudio del perfil de ácidos grasos de aceites de olivas virgen extra procedentes de diferentes variedades como indicador de su calidad nutricional y su estabilidad oxidativa.
2. Determinación de la estabilidad por oxidación térmica de los diferentes aceites de oliva procedentes de diferentes variedades mediante calorimetría diferencial de barrido.
3. Identificación de la temperatura de inicio de oxidación y el tiempo de inducción a la oxidación como indicadores de la estabilidad térmica de cada aceite a diferentes temperaturas.

4. Evaluación de la presencia de productos primarios y secundarios derivados de la oxidación del aceite de oliva por espectrofotometría ultravioleta determinando los coeficientes específicos de extinción ultravioleta.

OBJECTIVES

Nowadays, Spain is the major olive oil producer worldwide, with an average production in the last ten campaigns (2009/10 - 2018/19) of 1.34×10^6 tons per year. Consequently, this high production has triggered a series of challenges with respect to the wastewaters generated during the olive oil extraction process and the authentication of the quality of the olive oil produced.

First, olive oil industry generates large volumes of olive oil mill wastewaters (OMWs), which include olive and olive oil washing wastewaters. These wastewaters are characterized by their high organic load content as well as their high concentration of suspended solids, residual oil, etc. These facts make OMWs treatment complex and result in a great environmental impact. Currently, the most widespread practice for OMWs treatment is the management of these wastewaters through their accumulation in large reservoirs, for water evaporation during the summer months. Nevertheless, this solution results in numerous problems such as groundwater contamination, bad odours, etc. For this reason, seeking new treatments for these wastewaters is highly relevant to reduce environmental impact and to be able to reuse them.

Second, olive oil consumption is increasing worldwide due to its numerous nutritional and health benefits. These attributes are mainly determined by olive oil chemical composition, which can be altered because of oxidation processes, thermal processing or incorrect practices. In addition, it is highly relevant the correct labelling of olive oil as well as avoiding its adulteration with other lower quality oils. In this sense, the search for new fast, precise and economic techniques that guarantee the quality of olive oil could represent a great advance for the olive oil industry.

The specific objectives of this Doctoral Thesis are described below divided into chapters:

2.1. Integrated process for olive oil mill wastewater treatment and its revalorization through the generation of high added value algal biomass.

1. Physicochemical characterization of industrial wastewaters from the olive oil industry.

2. Design of a bioprocess for OMW treatment involving a primary physicochemical and secondary microalgal culture treatment. For the physicochemical treatment, the following operations were combined:
    i. Flocculation-sedimentation.
    ii. Photolysis by artificial UV-lamps.
    iii. Microfiltration.
3. Determination of the removal percentages for each operation unit and the quality of the final treated wastewater.
4. Use of OMW after primary treatment, at different concentrations, as culture medium for *Chlorella pyrenoidosa* growth.
5. Kinetic study of *Chlorella pyrenoidosa* cultures through the determination of the maximum specific growth rates and biomass productivities.
6. Determination of the biochemical composition of the harvested microalgal biomass, in terms of proteins, lipids and carbohydrates.
7. Evaluation of the effectiveness of the process designed for OMW bioremediation.

2.2. Combination of physicochemical operations and algal culture as a new bioprocess for olive mill wastewater treatment.

1. Physicochemical characterization of OMW.
2. Design of a bioprocess involving physicochemical treatment (as primary) and microalgal culture (as secondary) for OMW treatment. The primary treatment consisted of:
    i. Flocculation-sedimentation.
    ii. Microfiltration.
3. Determination of the emoval percentages for each operation unit and the quality of the final treated wastewater.
4. Use of OMW after primary treatment, at different concentrations, as culture media for the microalga *Scenedesmus obliquus.*
5. Kinetic study of *Scenedesmus obliquus* cultures through the determination of the maximum specific growth rates and biomass productivities.

6. Determination of the biochemical composition of the harvested microalgal biomass, in terms of proteins, lipids and carbohydrates.
7. Evaluation of the effectiveness of the process designed for OMW bioremediation.
8. Establishment of the best operating conditions in terms of microalgal growth, biodiesel production and pollutants removal.

2.3. Cultivation of *Scenedesmus obliquus* in mixtures of urban and olive oil mill wastewaters for the dual application of algal biomass production and wastewater treatment.

1. Physicochemical characterization of urban wastewater and olive oil mill wastewater.
2. Use of urban wastewater, as well as mixtures of urban and olive oil mill wastewaters (OMW pretreated by flocculation-sedimentation and UV photolysis) as culture media for *Scenedesmus obliquus*.
3. Kinetic study of *S. obliquus* growth in the mentioned culture media through the determination of the maximum specific growth rates and biomass productivities.
4. Determination of the biochemical composition of the harvested microalgal biomass, in terms of proteins, lipids and carbohydrates.
5. Evaluation of the bioremediation treatment during and at the end of *S. obliquus* cultures.
6. Global evaluation of the final quality of the treated wastewater and determination of the achieved removal percentages.

2.4. Determination of the Thermal Oxidation Stability and the Kinetic Parameters of Commercial Extra Virgin Olive Oils from Different Varieties.

1. Study of the fatty acids profiles of four extra virgin olive oils from different varieties as an indicator of their nutrition quality and oxidative stability.
2. Determination of the thermal oxidation stability of the olive oils by differential scanning calorimetry.
3. Evaluation of the oxidation onset temperatures and oxidation induction times as indicators of the thermal oxidation stability of each olive oil at different temperatures.

4. Study of the presence of primary and secondary products derived from the oxidation of olive oil through the determination of the specific UV extinction coefficients by UV spectrophotometry.

3. MARCO TEÓRICO/THEORETICAL FRAMEWORK

La industria oleícola es de vital importancia en España, el mayor productor y exportador de aceite de oliva a nivel mundial. Según el Consejo Oleícola Internacional, la producción media mundial de aceite de oliva durante el periodo 2009-2019 fue de $2{,}98\times10^6$ toneladas por año, produciéndose en España una media de $1{,}34\times10^6$ toneladas en el mismo periodo. Además, la producción de aceite de oliva tiende a aumentar cada año a nivel mundial debido a sus numerosos beneficios para la salud, derivados de su composición en ácidos grasos y antioxidantes naturales.

La elevada producción de aceite de oliva en todo el mundo ha provocado que la correcta gestión y reutilización de los residuos generados en las almazaras sea cada vez más urgente debido a la gran expansión de esta industria, así como el crecimiento de la preocupación sobre la protección del medio ambiente y el cambio climático. Por otro lado, otro de los retos a los que se enfrenta la industria oleícola es el establecimiento de métodos efectivos para la correcta caracterización del aceite de oliva para evitar así prácticas fraudulentas tales como su adulteración con otros aceites de menor calidad y coste.

En primer lugar, las aguas residuales constituyen el residuo más abundante en la almazara, generándose volúmenes de hasta $5{,}4\times10^6$ m^3 a nivel mundial en la campaña 2014/2015. Las características fisicoquímicas de las aguas residuales de almazara (ARA) dependen del proceso empleado para la extracción del aceite de oliva, pudiendo diferenciar entre el proceso discontinuo de prensa y el continuo por centrifugación. A su vez, el proceso continuo puede realizarse empleando un decánter con tres salidas (aceite, orujo y alpechín) o dos salidas (aceite y orujo húmedo). En España se utiliza actualmente el proceso de centrifugación más moderno, empleando un decánter de dos salidas, el cual da lugar a unas ARA con menos carga orgánica que las que se generan en el proceso de tres salidas (DQO = 40-200 g O_2/L). Aun así, se generan aguas residuales de lavado de aceitunas con menor carga orgánica (DQO = 0,5-1 g O_2/L) y aguas de lavado de aceite con mayor carga orgánica (DQO = 1-30 g O_2/L). La materia orgánica de estas ARA suele contener compuestos fenólicos, que son antioxidantes que actúan como compuestos inhibidores/tóxicos del crecimiento de los microorganismos, plantas y organismos acuáticos.

Por lo tanto, debido a la complejidad de sus características fisicoquímicas y los graves efectos que pueden tener tanto en ecosistemas acuáticos como terrestres, el tratamiento de las aguas

residuales de almazara debe ser obligatorio antes de realizar vertidos a otras aguas, de forma que se puedan reutilizar en otras actividades como el riego o en el propio proceso de fabricación. Actualmente, el almacenamiento de las ARA en balsas de evaporación es el método más extendido para su gestión debido a su sencilla construcción. Sin embargo, este sistema puede provocar contaminación de las aguas subterráneas mediante infiltración de las ARA, así como fuertes olores que atraen insectos.

En este sentido, se pueden encontrar en la bibliografía diferentes propuestas para el tratamiento de las ARA. En primer lugar, hay que destacar los tratamientos biológicos. Dentro de este grupo, la gran mayoría de estudios se centran en el desarrollo de procesos de digestión anaerobia. Sin embargo, este sistema requiere la eliminación previa de los compuestos fenólicos presentes o la realización de múltiples diluciones para una degradación más efectiva durante el proceso de digestión. En cuanto al tratamiento mediante procesos aerobios, han demostrado no ser efectivos con las ARA ricas en materia orgánica, requiriendo también múltiples diluciones previas. Por último, el co-compostaje de las ARA con diferentes fuentes de biomasa ha demostrado ser eficaz en la reducción de compuestos fenólicos, sin embargo, se trata de un proceso que requiere un elevado tiempo y no permite la recuperación de energía. Por otro lado, se encuentran las tecnologías de membrana (micro-, ultra- y nano- filtración, así como la ósmosis inversa), que podrían presentar una solución adecuada para el tratamiento de las ARA si se pudieran superar los desafíos derivados de la reducción de caudal debido al fouling en las membranas, además de la corta vida útil de las membranas, lo que provoca el incremento de los costos de tratamiento. En cuanto a los tratamientos termoquímicos, se han estudiado diferentes procesos tales como la gasificación supercrítica hidrotérmica o la gasificación catalítica en condiciones supercríticas. Sin embargo, estos requieren de unas condiciones muy severas y suponen un elevado coste.

Las microalgas son microorganismos fotosintéticos que se caracterizan por su facilidad de cultivo, rápido crecimiento y elevada productividad. Además, son capaces de crecer requiriendo únicamente agua, sales inorgánicas, CO_2 y luz solar, dando lugar a una biomasa con un elevado valor añadido por su composición rica en multitud de compuestos bioactivos que pueden ser empleados en diversas industrias como la farmacéutica, cosmética, alimentación, etc. Es por ello,

que el empleo de microalgas para el tratamiento de aguas residuales ha sido ampliamente estudiado debido a la capacidad de ciertas especies para degradar compuestos fenólicos, pesticidas, etc. En la bibliografía, se pueden encontrar diversos estudios empleando diferentes tipos de aguas residuales (municipales, agrícolas, industriales, etc.) como medio de cultivo de diferentes especies de microalgas.

En este trabajo de investigación se ha propuesto un proceso integrado para el tratamiento de aguas residuales de almazara que combina un tratamiento fisicoquímico con un tratamiento biológico basado en el uso de microalgas. En primer lugar, se incluyó una etapa de floculación-sedimentación, seguida de una etapa de fotólisis con luz ultravioleta artificial y una etapa de microfiltración con membrana, conectadas con el cultivo microalgal. Con este proceso se ha logrado un tratamiento eficaz de las ARA mediante un proceso que permite la generación simultánea de una biomasa de alto valor añadido que puede emplearse en diversas aplicaciones como la producción de biocombustibles.

Además, se ha propuesto la combinación de ARA pretratadas mediante el tratamiento fisicoquímico mencionado (floculación-sedimentación y fotólisis UV) con agua residual urbana (ARU) para la formación de un medio completo con todos los nutrientes necesarios para el crecimiento microalgal. Con este proceso se busca el establecimiento de un nuevo sistema que permita el tratamiento simultáneo de ambos tipos de aguas residuales, así como la generación de biomasa con alto valor añadido.

En este sentido, respecto a los métodos de tratamiento existentes para las aguas residuales urbanas, el más extendido es el sistema convencional. Este sistema combina operaciones fisicoquímicas y biológicas para mejorar la calidad del agua y se lleva a cabo en estaciones depuradoras de aguas residuales. El proceso consta de cuatro etapas: tratamiento preliminar, primario, secundario y terciario. A su vez, se pueden encontrar en la bibliografía nuevos métodos avanzados para el tratamiento de dichas aguas. Entre ellos podemos encontrar la ozonización, durante la cual se generan subproductos no deseados, así como productos de la oxidación. Además, su utilización requiere de una etapa posterior que elimine los subproductos orgánicos. También se pueden encontrar diversos estudios que emplean carbón activo, tanto granulado como en polvo.

Sin embargo, para la fabricación del carbón activo es necesaria una elevada cantidad de energía, además, éste pierde capacidad de adsorción con cada uso y regeneración. Por último, hay que destacar las tecnologías de membrana, especialmente la nanofiltración y la ósmosis reversa. Sin embargo, este sistema requiere de elevadas cantidades de energía, así como de unos costes muy elevados de inversión y reinversión. Además, da lugar a la generación de residuos muy concentrados y requiere de unas etapas previas que elimine la gran parte de los sólidos totales presentes en las ARA.

Además, en la presente Tesis Doctoral se aborda el problema referente al establecimiento de métodos efectivos para la caracterización y determinación de la calidad de los aceites de oliva. En este sentido, el Consejo Internacional Oleícola, la única Organización Internacional Intergubernamental del mundo en el sector del aceite de oliva y de las aceitunas de mesa, define la calidad de los aceites de oliva en función de cuatro parámetros: la acidez, el índice de peróxidos, los coeficientes específicos de extinción (K_{232} and K_{270}) y la calificación sensorial.

La acidez se define como el contenido en ácidos grasos libres en función del ácido oleico libre presente en el aceite. Las grasas producidas biológicamente son neutras, por lo que la presencia de ácidos grasos libres indica una anomalía derivada del mal estado de las aceitunas, así como procesos inadecuados de conservación. Este parámetro se calcula utilizando el método convencional de valoración, que consiste en disolver la muestra en una mezcla de disolventes y medir los ácidos grasos libres mediante análisis volumétrico utilizando una solución etanólica de hidróxido de potasio. Respecto al índice de peróxidos, éste mide el estado de oxidación inicial de un aceite, expresado como miliequivalentes de oxígeno activo por kilo de grasa. Este parámetro se mide disolviendo la muestra en ácido acético y cloroformo, posteriormente se trata con una solución de yoduro de potasio y el yodo liberado se titula con una solución de tiosulfato de sodio. Respecto a los coeficientes específicos de extinción, K_{232} y K_{270} son medidas espectrofotométricas para cuantificar la absorción UV a 232 y 270 nm, respectivamente. Éstos proporcionan información sobre la calidad de la grasa y el estado de conservación del aceite y para su medida se emplea ciclohexano como disolvente y se mide la absorbancia a las longitudes de onda mencionadas.

A parte de los métodos mencionados, se pueden encontrar en la bibliografía otros adicionales para la caracterización y determinación de la calidad de los aceites de oliva. Entre ellos cabe mencionar el método Estabilidad Rancimat, que consiste en exponer el aceite a una temperatura de 100ºC con inyección de aire para forzar su oxidación. A esta temperatura, los ácidos volátiles de cadena corta se transportan a otro recipiente que contiene agua destilada que se mide su conductividad de forma continua. La conductividad eléctrica de esta agua aumenta con la presencia de los ácidos volátiles. El tiempo necesario para producir un aumento brusco de la conductividad define la estabilidad del aceite. Por otro lado, en los últimos años, la espectrofotometría infrarroja ha emergido como técnica para el estudio de la estructura de los componentes alimentarios, así como para el seguimiento de su calidad. Estas técnicas (espectroscopia de infrarrojo cercano y medio) no requieren complejos pretratamientos de la muestra ni análisis químicos destructivos y complejos, ni grandes cantidades de disolventes orgánicos. Sin embargo, debido a la heterogeneidad del aceite de oliva, la detección/determinación de compuestos minoritarios es difícil. Por último, mencionar la espectroscopía de resonancia magnética nuclear (RMN), ampliamente empleada para el análisis del aceite y que se ha establecido recientemente como una valiosa herramienta para la evaluación de la calidad y autenticidad del aceite de oliva. Además, esta técnica permite la detección de aceites de oliva adulterados. El inconveniente de esta técnica se encuentra en la interpretación de la gran cantidad de datos que proporcionan las señales de RMN, que requiere de un sistema adicional para su procesamiento.

En esta Tesis, se ha propuesto el empleo de la técnica de calorimetría diferencial de barrido como método para evaluar la estabilidad oxidativa y la calidad del aceite de oliva. A pesar de que esta técnica aún no está recogida por el Consejo Internacional Oleícola como un método oficial, ha demostrado ser eficiente, rápida, precisa y respetuosa con el medio ambiente, puesto que no requiere el uso de solventes o el pretratamiento de la muestra. Además de la calorimetría diferencial de barrido, se han empleado otras técnicas que han permitido la caracterización química de las diferentes variedades de aceite de oliva, así como el estudio de su calidad. Por un lado, se ha empleado la cromatografía de gases para la determinación del perfil de ácidos grasos, que constituye un indicador tanto de la calidad nutricional como de la estabilidad oxidativa del aceite de oliva. Por

otro lado, el empleo de espectrofotometría ultravioleta ha permitido estudiar la presencia de productos derivados de la oxidación de los aceites de oliva.

THEORETICAL FRAMEWORK

Olive oil industry is of vital importance in Spain, the world's leading producer and exporter of olive oil. According to the International Olive Oil Council, the average world production of olive oil during the period 2009-2019 was 2.98×10^6 tonnes per year, with Spain producing an average of 1.34×10^6 tonnes during the same period. Furthermore, olive oil production is increasing worldwide due to its numerous nutritional and health benefits, derived from its composition in fatty acids and natural antioxidants.

The correct management and reuse of olive mills wastes has become highly urgent due to the great expansion of this industry as well as the growing concern for the environment protection and climate change. In addition, another challenge that must be faced nowadays by the olive oil industry is the establishment of effective methods for the proper characterization and identification of olive oil to avoid fraudulent practices such as adulteration with lower quality oils.

Regarding the wastes generated by the olive oil industry, olive oil mill wastewaters (OMWs) constitute the most abundant residue, reaching values of up to 5.4×10^6 m^3 of OMW worldwide in the 2014/2015 campaign. OMW physicochemical characteristics depend on the olive oil process used for its extraction, which can be performed throughout discontinuous (press) or continuous (centrifugation) processes. Continuous centrifugation processes can be performed using a decanter (horizontal centrifuge) with two or three outlets. In Spain, the most modern centrifugation process (decanter with two-exits) is currently used, which results in OMWs with less organic load than those generated in the three-exit process (COD = 40-200 g O_2/L). Even so, olives washing wastewater with a lower organic load (COD = 0.5-1 g O_2/L) and oil washing wastewater with a higher organic load (COD = 1-30 g O_2/L) are generated. The organic matter in these OMWs usually contains phenolic compounds (natural antioxidants) that act as inhibitory/toxic compounds to the growth of microorganisms, plants and aquatic organisms.

Therefore, due to the complex physicochemical characteristics and the serious impact that OMW have on aquatic and terrestrial ecosystems, the treatment of these wastewaters is obligatory before being discharged into receiving waters or reused in other activities such as irrigation.

Nowadays, the storage of OMWs in evaporation reservoirs is the most widespread method used for their management due to their simple constructions. Nevertheless, this system can provoke the contamination of groundwater by infiltrations as well as bad odors and insect proliferation. In this sense, different proposed treatments for OMW can be found in the bibliography. Biological treatments are the most prominent. Numerous studies are focused on the development of aerobic and anaerobic digestion processes. However, these systems require the prior removal of phenolic compounds or the performance of multiple dilutions for a more effective degradation during the biodegradation processes. Aerobic treatments have been proven ineffective with OMW due to its high organic load. Co-composting of OMWs with different biomass sources has been shown to be effective in phenolic compounds removal, nevertheless, a high time-consuming process is required in addition to the low energy recovered. Membranes technologies (micro-, ultra- and nano-filtration) could be an effective treatment method if the fouling problems could be overcome and the costs were reduced. With respect to thermochemical treatments, some processes such as hydrothermal supercritical gasification or catalytic gasification under supercritical conditions have been studied. However, these processes are performed under severe energetic conditions and represent a high cost.

Microalgae are photosynthetic microorganisms characterized by their easy culture, rapid growth and high productivity. Furthermore, microalgae can grow requiring only water, inorganic salts, CO_2 and sunlight, generating a high added value biomass rich in bioactive compounds that can be used in numerous industries such as pharmaceutical, cosmetics, food, etc. For this reason, the use of microalgae in wastewaters treatment has been widely studied due to the ability of certain species to degrade phenolic compounds, pesticides, etc. Numerous studies can be found in the bibliography in which different types of wastewaters (municipal, agricultural, industrial, etc.) are used as microalgae culture media.

In this Doctoral Thesis, it has been proposed an integrated process for the treatment of OMWs that combines physicochemical and biological operations. Firstly, it was performed a primary treatment based on flocculation-sedimentation operation followed by UV photolysis and membrane microfiltration. Secondly, a microalga culture as secondary treatment. This process

allowed the efficient OMW treatment and, at the same time, the generation of microalgal biomass with high added value, which can be subsequently used for biofuels production.

Furthermore, it has been proposed the combination of pretreated OMWs throughout the mentioned physicochemical treatment (flocculation-sedimentation and UV photolysis) with urban wastewater (UW), with the aim of achieving a complete medium (with all nutrients required) for microalgae growth. This process seeks to establish a new system for the simultaneous treatment of both wastewaters as well as the generation of high added value biomass.

With regard to the existing methods for UW treatment, the most widely used is the conventional system, which is performed in sewage treatment plants and combines physicochemical and biological operations to improve the final water quality. This process consists of four steps: preliminary, primary, secondary and tertiary treatment. Additionally, new advanced methods for UW treatment can be found in the bibliography. These include ozonation, during which unwanted by-products such as oxidation products are generated. Furthermore, the application of this process requires a subsequent step to remove the organic by-products. Several studies can also be found in which active carbon (both granulated and powdered) is used for UW treatment. However, its production needs high energy and its adsorption capacity decreased with each use. Finally, membrane technology has been widely used in the last two-decades, especially nanofiltration and reverse osmosis. Nevertheless, this technology has high-energy requirements and high maintenance and investment costs. In addition, this technology generates a concentrated rejection that must be treated.

In addition, this Doctoral Thesis has addressed the issue of establishing effective methods for the proper identification of olive oil and the determination of its quality. In this sense, the International Olive Council, the only international organization in the field of olive oil and table olives, defines the quality of olive oil according to four parameters: free acidity, peroxide value, UV specific extinction coefficients (K_{232} and K_{270}) and sensory evaluation.

The free acidity is defined as the content of free fatty acids expressed as oleic acid. The high presence of free fatty acids is a resulting anomaly, among other factors, of the poor state of the

fruits as well as inadequate treatment and conservation processes. This parameter is determined using the conventional method of titration, which involves dissolving the sample in a solvent mixture and measuring the free fatty acids by volumetric analysis using an ethanolic solution of potassium hydroxide. Regarding the peroxide value, this measures the amount of peroxide that causes the oxidation of potassium iodide, expressed in milliequivalents of active oxygen per kilogram of fat. This parameter is measured by dissolving the sample in acetic acid and chloroform, then, it is treated with a potassium iodide solution and the liberated iodine is titrated with a sodium thiosulfate solution. Finally, with respect to the UV specific extinction coefficients, K_{232} and K_{270} values are spectrophotometric measures for quantifying the UV absorption at 232 and 270 nm, respectively. It provides information about the quality of the fat and the conservation status of the oil, for its measurement it is used cyclohexane as solvent and the absorbance of the solution is measured at the specified wavelengths.

In addition to the official methods defined by the International Olive Council, other techniques for identification and determination of olive oil quality can be found in the bibliography. These include the Rancimat Stability method, which consists of exposing the olive oil to forced oxidation by air at 100ºC until its maximum oxidation. At this temperature, the short-chain volatile acids that are formed and transported to a distilled water solution cause an increase in the electric conductivity. The time needed to produce a sharp increase in the electric conductivity defines the stability of the olive oil. On the other hand, infrared spectroscopy has emerged as a technique for studying the structure of food components and monitoring their quality. These techniques (near- and mid-infrared spectroscopy) do not require complex sample pretreatments, destructive and complex chemical analysis or large volumes of organic solvents. However, due to olive oil heterogeneity, the detection/determination of minority compounds is difficult. Finally, it should be mentioned the nuclear magnetic resonance spectroscopy (NMR), that has been widely used in olive oil analysis and recently established as a valuable technique for the evaluation of olive oil quality and authenticity. This technique allows the detection of adulterated olive oils. The drawback of this method lies in the interpretation of large amounts of data provided by NMR signals, which requires an additional system for their processing.

In this Doctoral Thesis, differential scanning calorimetry (DSC) has been proposed as a technique for the evaluation of olive oil oxidative stability. Although the International Olive Council has not defined DSC as an official method, it has proven to be effective, fast, precise and environmentally friendly, since it does not require the use of solvents or sample pretreatment. In addition, other techniques have been used for the chemical characterization of the different olive oil varieties as well as the determination of their authenticity. On the one hand, gas chromatography was used to determine the fatty acids profiles, as an indicator of the nutritional quality and the oxidative stability of olive oil. On the other hand, the use of ultraviolet spectrophotometry allowed to study the presence of initial and final oxidation products.

4. RESULTS AND DISCUSSION

4.1. INTEGRATED PROCESS FOR OLIVE OIL MILL WASTEWATER TREATMENT AND ITS REVALORIZATION THROUGH THE GENERATION OF HIGH ADDED VALUE ALGAL BIOMASS

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ABSTRACT

The two-phase continuous centrifugation process for olive oil extraction generates high amounts of olive oil mill wastewater (OMW), characterized by containing large concentrations of numerous contaminant compounds for the environment. An integral process based on physico-chemical (flocculation, photolysis and microfiltration) and microalgal growth stages was proposed for its treatment. Chemical oxygen demand (COD) removal percentages were 57.5%, 88.8% and 20.5% for flocculation, photolysis and microfiltration, respectively. The global removal percentages of organic load in the primary treatment were 96.2% for COD, 80.3% for total organic carbon (TOC) and 96.6% for total phenolic compounds (TPCs). In secondary treatment, different experiments using the microalgae *Chlorella pyrenoidosa* were performed on a laboratory scale in stirred batch tank reactors. The OMW concentrations in each culture medium were: 5%, 10%, 25%, 50%, 75% and 100% (v/v). The common experimental conditions were: pH = 7, temperature = 25ºC, agitation speed = 200 rpm, aeration rate = 0.5 (v/v) and illumination intensity = 359 $\mu E\ m^{-2}\ s^{-1}$. The highest maximum specific growth rate (0.07 h^{-1}) and volumetric biomass production (1.25 mg/(L h)) values were achieved in the culture with 50% of OMW (v/v). The final biomass obtained had a high percentage of carbohydrates, whose content ranged from 30.3% to 89.2% and the highest lipid content (34.2%) was determined in the culture with 25% of OMW (v/v). The final treated water is suitable for its use in irrigation, discharge to receiving waters or for being reused in the same process.

Keywords: Olive oil mill wastewater; Flocculation; Photolysis; Biomass growth; *Chlorella pyrenoidosa*; Treatment.

4.1.1. Introduction

Microalgae are photosynthetic microorganisms that are characterized by its easy culture, and high growth and productivity rates. These microorganisms produce biomass with high-added value products as pharmaceutical compounds, fatty acids, carotenoids, dyes and fine chemicals. All these compounds can be used for human, animal and aquatic feed (Hodaifa et al., 2013; Mata et al., 2010; Nor et al., 2016; Rawat et al., 2011; Suganya et al., 2016). On the other hand, they are able to grow in harsh conditions requiring water, inorganic salts, CO_2 and sunlight (Mata el al., 2010). In this sense, microalgae have numerous environmental applications such as CO_2 mitigation and wastewater treatment (Suganya et al., 2016). Furthermore, certain species have the capacity to degrade a large variety of compounds such as xenobiotic, polyaromatic hydrocarbons, phenolic compounds, pesticides, etc. For all these reasons, the dual application of microalgae for wastewater treatment and biomass production is an attractive alternative with great industrial and economic potential (Hodaifa et al., 2012; Rawat et al., 2011).

Different wastewaters such as municipal, agricultural and piggery have been used as microalgae culture media for nutrient removal and biomass production (Abou-Shanab et al., 2013; Ji et al., 2014; Mata el al., 2010; Rawat et al., 2011). Many works have shown the ability of microalgae to degrade and remove excess nutrients (mainly persistent and hazardous organic pollutants) in wastewaters. The capacity of *Chlorella, Ankistrodesmus* and *Scenedesmus* species to remove contaminants from olive oil mill and paper industry wastewaters has already been demonstrated (Hodaifa et al., 2012 and 2013; Kouhia et al., 2015). In general, wastewaters have a complex physicochemical composition, for this reason, the nutrient availability and the presence of growth inhibitors could influence microalgal growth (Guldhe et al., 2017; Hodaifa et al., 2012).

Olive oil industry is an important sector within the agro-food industries in the Mediterranean countries (Hodaifa et al., 2012) as well as in non-traditional producing countries (as Australia, New Zealand and South America) due to the growing interest in olive oil consumption and production. Olive oil is obtained from olive fruit by mechanical procedures throughout pressing (discontinuous) and centrifugation systems (continuous). The last systems can be carried out by using a 'Decanter' with two or three exits (Dermeche et al., 2013). In Spain, the main olive oil

producer worldwide, the centrifugation process using a 'Decanter' with two exits (for olive oil and pomace production) is currently used (Tsagaraki et al., 2007). Olive oil mill wastewater (OMW) from two exits is characterized by containing a high concentration of organic matter which includes polysaccharides, sugars, phenolic compounds, polyalcohol, nitrogenous compounds, organic acids, tannins, pectin, lignin, oil and high levels of suspended solids (Dermeche et al., 2013; Mantzavinos and Kalogerakis, 2005). In this sense, OMW produced by 'Decanter' with two exits have less organic load (COD = 4-16 g O_2/L) in comparison with the wastewaters generated using a 'Decanter' with three exits or the pressing process (COD = 40-220 g O_2/L), (Agabo-García and Hodaifa, 2017).

In this work, a new process for real OMW treatment based on physico-chemical operations (as primary treatment) followed by microalgae culture (as secondary treatment) was proposed. First operations included flocculation-sedimentation, photolysis and microfiltration units connected with *Chlorella pyrenoidosa* culture. In this sense, physico-chemical characteristics of the real crude olive oil mill wastewater were studied. Flocculation-sedimentation and photolysis operations were established and optimized. Then, different dilutions of primary treated OMW (5%, 10%, 25%, 50%, 75% and 100% v/v) were used as culture media. Kinetic growth, biomass production and biochemical composition of *C. pyrenoidosa* were evaluated. Treated water and bioremediation of the wastewater during the integral process were determined.

4.1.2. Experimental

4.1.2.1. Microorganism and photobioreactor

The microorganism used was the freshwater green algae *Chlorella pyrenoidosa* Chich 8H Emerson. Experiments were performed in sterile conditions, on a laboratory scale in stirred batch tank reactors with work capacity = 1 L, diameter = 10 cm and height =16 cm. All bioreactors had continuous illumination on one side.

4.1.2.2. Procedure

OMW was obtained from an olive oil extraction plant in the province of Seville (Spain). The flocculation-sedimentation was carried out during 90 min in Imhoff funnel using a commercial

flocculant Flocudex CS-51. Based on a previous study (Hodaifa et al., 2015) an optimal flocculant concentration of 100 mg/L was selected.

The obtained supernatant was subjected to photolysis in a batch stirred photoreactor with total capacity equal to 750 cm^3 (work volume = 600 cm^3). A commercial medium pressure UV immersion lamp, model TQ 150 Brand HNG Germany G4, 150 N° 5600 1725 (Standard) was used. During the proposed process the reduction of organic matter was determined.

Culture media were prepared by mixing OMW and ultrapure water to obtain the following final concentrations: 5%, 10%, 25%, 50%, 75% and 100% (v/v) OMW. Sterilization was performed by filtration through a membrane with pore size equal to 0.2 μm.

The pH was adjusted and maintained at a value of 7.0 over the course of the culture through the addition of 0.1 mol NaOH L^{-1} or 0.1 mol HCl L^{-1} solution.

The common culture conditions were: temperature = 25ºC, aeration rate = 0.5 L min^{-1}, pH value = 7, magnetic agitation speed = 200 rpm and continuous light with illumination intensity equal to 359 μE m^{-2} s^{-1}.

In all the experiments, the precultures of *C. pyrenoidosa* were grown for seven days at room temperature in solidified Rodríguez-López medium (Rodríguez-López, 1964) with agar at 2% (w/w) under continuous illumination. The liquid inoculum (0.0141±0.00791 g/L) for each experiment consisted of a suspension of cells in sterile Rodríguez-López culture medium.

4.1.2.3. Microalgae growth

The biomass concentration, x g L^{-1}, was measured indirectly by the absorbance of the cell suspension in ultrapure water at 600 nm (Camacho et al., 1989) after two centrifugation stages in which biomass was washed with ultrapure water. Results obtained allowed the representation of growth curves and the determination of the growth kinetic velocities.

The specific growth rate ($\mu = 1/x \cdot dx/dt$) in the exponential phase and the biomass productivity ($P_b = dx/dt$) in the linear phase were determined.

4.1.2.4. Biochemical composition of the biomass

In all experiments, the total pigments (total chlorophylls and total carotenoids) were determined during the course of the cultures. At the end of each experiment, algal biomass was separated, and total lipids, proteins and fatty-acids contents were determined.

Total lipids were obtained by using a micro-soxhlet extractor with n-hexane as solvent. Fatty acid profile was determined and identified directly from dried algal biomass by gas chromatography using a Hewlett–Packard, Model 5890 Series II equipped by a FID detector (Lepage and Roy, 1984). The crude protein content was performed from the nitrogen percentage determination (%Crude proteins = %TN×6.25, Becker, 1994) using a Total Carbon and Nitrogen Analyser provided by Skalar Company, mod. Formacs^{HT} and Formacs^{TN}.

The total carbohydrate content was obtained by considering that algal biomass is formed by proteins, carbohydrates, lipids, pigments and genetic material. For carbohydrate content calculation, genetic material was considered approximately about 1% (Becker, 1994).

4.1.2.5. Analytical methods

In the characterization of wastewater and treated water (crude and after each treatment), the following parameters were determined: pH value, electric conductivity, turbidity, chemical oxygen demand (COD), total phenolic compounds (TPCs), total organic carbon (TOC), total carbon (TC), inorganic carbon (IC), total nitrogen (TN), total iron, sulphates, sodium, ortho-phosphate and ammonium.

pH, electric conductivity (EC) and turbidity values were directly measured by using a pH-meter Crison, mod. GLP 22C, Conductimeter Crison, mod. GLP31 and Turbidimeter Hanna, mod. HI93703, respectively.

The determination of TPCs was carried out by making it react with a derivative thiazol, giving a purple azo dye, which was determined photometrically at 475 nm according to the standard methods (ISO 8466-1; DIN 38402 A51).

COD was determined photometrically at 620 nm according to German standard methods (DIN 38409 H41).

TOC, TC, IC and TN contents were determined using a Total Carbon and Nitrogen Analyser provided by Skalar Company, mod. Formacs^{HT} and Formacs^{TN}.

Total iron ions determination was performed through the reduction of all iron ions to iron (II) ions in a thioglycolate medium with a derivative of triazine. This reaction results in a reddish-purple complex that was determined photometrically at 565 nm according to the standard methods (ISO 8466-1; DIN 38402 A51).

Sulphates and ortho-phosphates were determined photometrically at 420 nm and 690 nm, respectively, according to the standard methods (ISO 8466-1; DIN 38402 A51).

Sodium, ammonium, potassium and calcium contents were determined directly by using selective ion electrodes for each one (Crison, mod. GLP 22C).

Finally, carbohydrate content (total reducing sugars) could be determined by using the DNS (dinitrosalicylic acid) method as described by Miller (1959). In this method, 3 mL of DNS reagent is mixed with 2 mL of sample. Then the sample is immersed in a water bath at 80-85 ºC for 5 min. After cooling to room temperature, the sample is measured photometrically at 540 nm. In addition, a calibration line using glucose as reference reagent is needed.

4.1.2.6. Calculation methods and reproducibility

In this work, experiments were made at least in duplicate and analytical methods were applied at least in triplicate. Models calculation and statistical methods used were available in the OriginPro 8.0 program.

4.1.3. Results and Discussion

4.1.3.1. Characterization of raw OMW used

Wastewater must contain a suitable nutrient profile for its use as culture medium for microalgae, with carbon, nitrogen and phosphorous sources as the most essential elements required for algal biomass growth. Table 1 shows the composition of raw and treated industrial olive oil wastewater used in this work. It is necessary to highlight the high presence of high organic matter, determined in terms of turbidity = 714 FTU, COD = 5839 mg O_2/L, TPCs = 322 mg/L, TOC = 646 mg/L and TN = 58.9 mg/L. The high TN concentration registered can be explained by the presence of proteins and other nitrogenated compounds in the OMW composition, which come from the olive fruit crushing and olive oil washing (Agabo-García and Hodaifa, 2017).

High concentrations of phenols (TPCs = 322 mg/L) were also found. These latter compounds have a similar structure to that of lignin, which makes them difficult to be biodegraded. They are also characterized by a high specific chemical oxygen demand, phytotoxicity and antibacterial activity, being the major contributors to the OMW toxicity and microalgal growth inhibition (Azabou et al., 2007; D'Antuono et al., 2014; Fountoulakis et al., 2002). A high inorganic salts portion was also detected (318 mg/L), as well as phosphorus in the form of inorganic salts (ortho-phosphate = 43.1 mg/L), which play an important role in microalgae cell growth and metabolism through phosphorylation reactions. On the other hand, it must be also indicated the high COD/TOC ratio value (equal to 9) registered for raw OMW in comparison with domestic wastewater in which this value is around 2 to 3 (Huang et al., 2010). Similarly, high COD/TOC values have been registered in several industrial wastewater studies. Güneş et al., (2019) described industrial container and drum cleaning wastewater (Sample 3) with COD/TOC = 6.21. Agabo-García and Hodaifa (2017) determined for crude wastewater from washing olives (WOW) a COD/TOC ratio = 8.12. Buthiyappan and Abdul Raman (2019) indicated COD/TOC ratio values from 9.41 to 11.2 for textile wastewaters and Dhanke et al. (2018) established COD/TOC ratio = 24.3 for fish processing industry wastewaters. This fact can be explained by the high heterogeneity of industrial wastewaters physicochemical characteristics, which is mainly determined by the wastewater origin (Raper et al., 2018).

The low iron content can be explained by the use of drinking water in food industries for washing raw materials. High iron concentration is not desired since it is a microalgae growth inhibitor (Fazal et al., 2018).

Table 1. Characterization of raw and treated OMW during treatment process.

Parameter	Raw OMW	Primary treatment			Secondary treatment		
		Physico-chemical sequence treatment			%Treated OMW after algal culture (v/v)		
		Flocculated	UV	Microfiltration	25	75	100
pH	8.25	Natural*	Natural	Natural	7.0	7.0	7.0
Conductivity, mS/cm	1.9	1.34	1.35	1.28	0.35	0.96	1.26
Turbidity, FTU	714	53.5	21.9	2.37	6.75	14.0	14.1
COD, mg O_2/L	5839	2484	279	222	-	58.5	138
TPCs, mg/L	322	70.9	38.5	10.8	0.911	3.09	7.39
TC, mg/L	1400	561	237	199	51.8	117	153
TOC, mg/L	646	530	149	127	31.2	69.2	147
TN, mg/L	58.9	27.8	22.4	17.3	2.15	5.22	5.65
IC, mg/L	318	31.3	87.5	71.9	20.6	47.5	26.5
Iron, mg/L	1.19	1.03	0.857	0.508	0.15	0.29	0.490
Sulphate, mg/L	320	84.8	79.8	52.3	15.8	29.3	51.8
Sodium, mg/L	0.943	0.782	0.168	0.208	-	-	0.120
Ortho-phosphate, mg/L	43.1	21.7	21.3	-	-	-	-
Ammonium, mg/L	4.44	4.09	1.32	-	0.14	0.18	0.310

** pH value of OMW without modifying.*

4.1.3.2. Bioprocess for olive oil mill wastewater treatment

The proposed new real OMW treatment process was performed according to Fig. 1. The process undertaken consisted of four phases, of which, the first three phases correspond to the primary treatment and the last stage, to the secondary treatment:

i) Flocculation-sedimentation. It was performed in two steps without pH modification. In the first, to complete mixing of flocculant and effluent, a high agitation speed (700 rpm) was applied for 1 min. In the second, to achieve flocs formation, a low agitation speed (350 rpm) for 30 min was employed. The aim of this stage was to separate and remove the solid fraction of OMW, which consisted of a sludge that can be used subsequently for composting. For this purpose, flocculated OMW was left to settle during 30 min.

ii) Photolysis. It consisted of the exposition of the obtained supernatant (after removal of the solid fraction) to UV-light for 30 min. The objective of this stage was the elimination of a part of the organic matter present in OMW, especially organic compounds as phenols, which are considered as microbial growth inhibitors. Sample settling during 30 min was performed to allow the sedimentation and subsequent separation of the remaining solid fraction.

iii) Microfiltration. It was used for OMW microbial (sterilization) and organic load reduction.

iv) *Chlorella pyrenoidosa* culture for the bioremediation of OMW and the obtaining of microalgal biomass with added value, mainly energetic compounds, which could be used for biofuels and biogas production or directly used in boilers for biomass combustion.

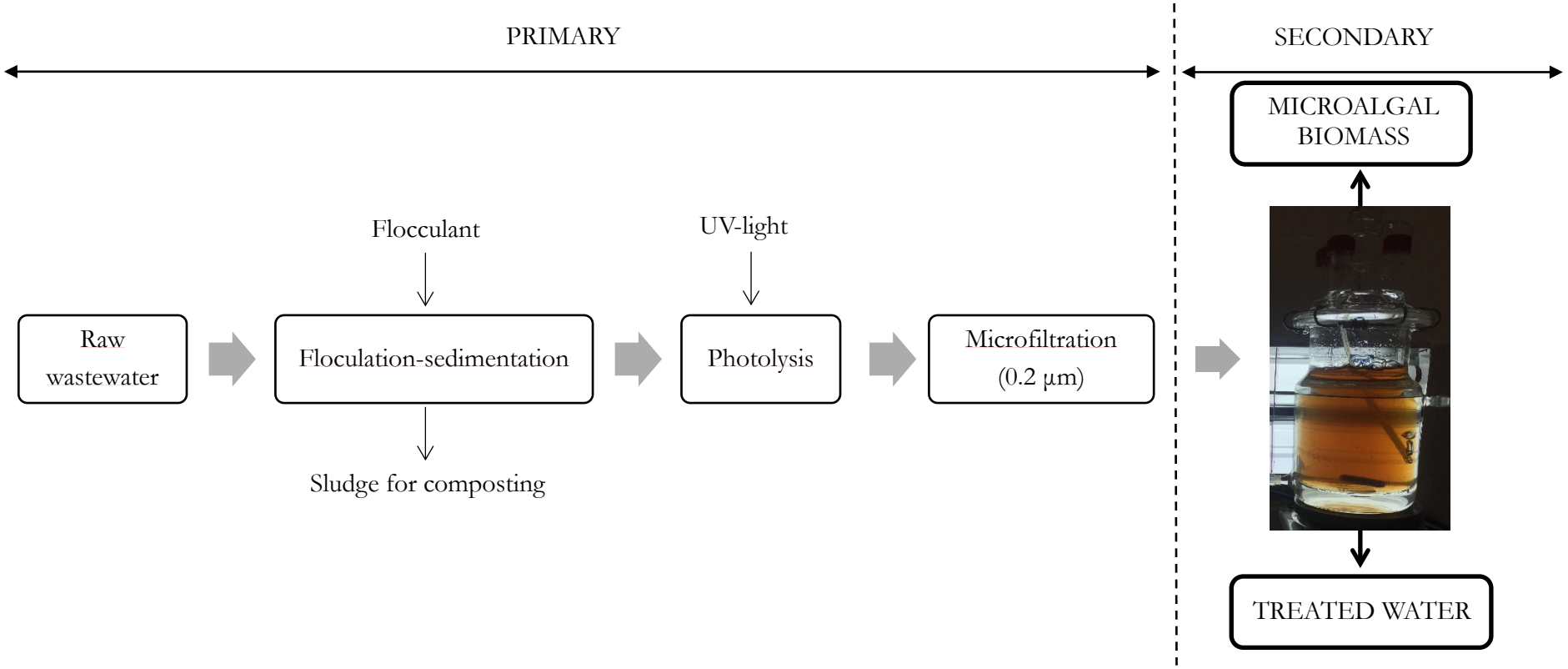

Fig. 1. Schematic representation of the proposed bioprocess for OMW treatment.

4.1.3.3. Primary treatment

Table 1 shows the variation of the treated water composition during the primary treatment. In general, all parameters were decreased throughout the primary treatment. Flocculation stage allowed a high total phenolic compounds removal percentage of up to 78% (Table 1). Theoretically, after the use of flocculant in OMW treatment, an increase in TPCs is expected due to the presence of phenolic compounds in the flocculant composition. The commercial Flocudex CS51 used is a solid cationic polyelectrolyte with high molecular weight and high capacity to eliminate suspended solids, turbidity and compounds responsible for colour apparition. In this sense, it is important to indicate that commercial flocculants usually incorporate a lignosulfonate, guaiacol (methoxy phenol) or protocatechuic acid in the synthesis process of acrylamide copolymers (He et al., 2015). After the photolysis operation, the TPCs concentration was decreased to 38.5 mg/L (%TPCs removal = 45.7%) due to the degradation process of lignin and phenols by the UV-light (El Hajjouji et al., 2007; Machado et al., 2000). Lignin polymer, which is largely present in olives pulp, is a natural polymer whose main structural units are phenolic compounds (Tanaka et al., 1999).

From the environmental point of view, the organic load can be determined by COD and TOC parameters. During the flocculation, photolysis and microfiltration the removal percentages 57.5%, 88.8% and 20.5% for COD and 18.0%, 71.9% and 14.6% for TOC were determined, respectively.

As a result of the flocculation process, the TOC/TN ratio increased from 11.0 (crude OMW) to 19.1 (flocculated OMW) indicating a strong fall in nitrogen content due to the efficient protein removal (component with high molecular weight) by the flocculant. After that, the ratio decreased to 6.66. This showed that during flocculation, a high percentage of proteins were removed and during photolysis, higher levels of organic matter oxidation were achieved. In general, the variation in the different determined ratios after flocculation does not follow a fixed pattern. In this sense, COD/TOC ratio was decreased from 9.04 to 4.69 through flocculation. This separation depends on the aggregation mechanism applied (charge neutralization, entrapment mainly by Van der Waals forces, adsorption forces, complexation with coagulant metal/flocculent ions into

insoluble particulate aggregates, Matilainen et al., 2010). Therefore, the separation mechanism through flocculation is a non-selective separation.

During microfiltration the TOC/TN ratio registered a slightly increase (7.4) indicating higher carbon compounds removal in comparison with the elimination of nitrogenated compounds.

In view of the results achieved, it can be confirmed that photolysis was the most effective operation for organic load reduction. Von Sonntag (2008) showed the effectiveness of UV-light for organic matter photodegradation in comparison with natural oxidation. Photolysis is a photochemical operation in which organic compounds are partially decomposed because of the absorption of this high-energy irradiation. Agabo-García and Hodaifa (2017) studied the UV-light effect in the degradation of OMW organic matter in photoreactors. They observed that photodegradation occurs in one step by an instantaneous reaction in the first minutes (< 4 min). Afterwards, no significant degradation was observed. In addition, Catalá et al. (2015) when using a 150 W medium pressure mercury lamp (The same UV-lamp used in this work) in natural fluvial waters containing illicit drugs achieved high TOC removal level equal to 79%.

This high elimination percentage obtained after photolysis is due to the special characteristics of UV-lamp used, wide emission range and high potency. In this case, a commercial medium pressure UV immersion lamp, model TQ 150 Brand HNG Germany G4, 150 W, Nº 5600 1725 (Standard) was used. In general, medium pressure mercury lamps are available in different potency from 100 to 1000W. The emission profile of these lamps consists on a wide range of wavelengths from 200 to 700 nm (UV and visible light) and the peak of 254 nm is strongly diminished. The emission intensity of these lamps is at least 10 fold higher than that of low-pressure arcs but happens on a much smaller surface. This UV-lamp type in contrast to other develops a considerable amount of heat, which cooling is required, but this problem can be resolved by running tap water to maintain the temperature around 20 ºC (Albini and Germani, 2010). In addition, this fact is not important when working at pilot or industrial plant since the reactor volume itself is enough to remove the heat generated by the UV-lamp.

Other authors have shown that artificial UV-light oxidation allows the rapid decomposition of toxic compounds such as nitrosodimetylamine (NDMA), hydrazine, 1,4-dioxane and methylthrethylbutaneethyl (MTBE), (McCurry et al., 2016; Radjenovic et al., 2012; Tawabini et al., 2013).

Sulphate ions were efficiently removed during the primary treatment (Table 1). High sulphate ions removal percentages (73.7% and 34.5%) were registered after flocculation and microfiltration, respectively. Sulphate ions elimination from water and wastewater is complex due to the high solubility and stability of these anions in aqueous solutions. The main methods used for its treatment are: (1) biological degradation, (2) membrane filtration (primarily reverse osmosis), (3) adsorption/ion exchange in resins, and (4) chemical precipitation (Amaral Filho et al., 2016).

4.1.3.4. Secondary Treatment (microalgal treatment)

i. *Chlorella pyrenoidosa* growth

Fig. 2A shows a sample of the growth curves of *C. pyrenoidosa* when the microalgae was grown in a 10% OMW (v/v) culture. In general, a short duration (<18 h) lag or adaptation phase was detected in all experiments. This phase was followed by an exponential growth phase whose duration ranged from 20 to 32 h in the cultures with %OMW<75% (v/v). Only in the case of 100% OMW (v/v) the duration of this phase was 61.5 h. Then, a deceleration growth phase with linear behaviour was observed. The duration of the linear growth was increased with the augment of %OMW in the culture medium (from 25 to 144 h). This appears to indicate that this phase is determined by the limitation of one or more nutrients. A stationary phase of growth at the end of the culture was observed in all experiments. In this sense, similar growth curves were obtained by Hodaifa et al. (2008, 2009, 2012) using OMW from two and three-phase systems as culture media for *Scenedesmus obliquus*.

The determination of the maximum specific growth rate and biomass productivity of *C. pyrenoidosa* were determined according equations (1) and (2), respectively (Fig. 2A).

$$ln\left(\frac{x}{x_o}\right) = \mu_m\, t + a \quad (1)$$

where 'μ_m' is the slope of the line and corresponds to the maximum specific growth rate and 'a' is the intercept.

$$x = P_b\, t + b \quad (2)$$

where 'P_b' is the slope of line and corresponds to the volumetric biomass productivity and 'b' is the intercept.

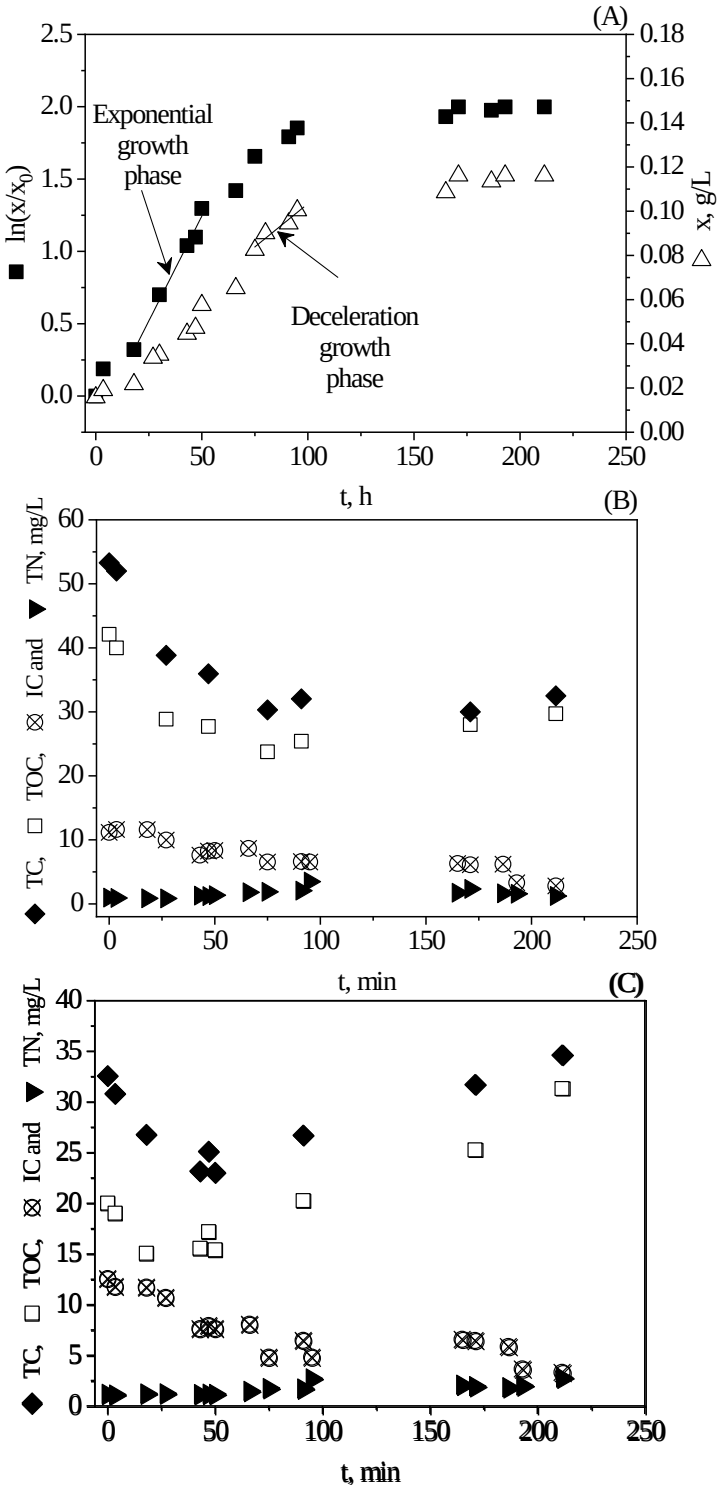

Fig. 2. *Chlorella pyrenoidosa* growth curves on 10% OMW. A) Determination of maximum specific growth rate and volumetric biomass productivity. B) Variation of total carbon species and total nitrogen on the global algal culture (algal biomass plus OMW). C) Variation of total carbon species and total nitrogen on the treated OMW (without algal biomass) during the culture.

It can be observed in Fig. 3 the variation of the maximum specific growth rates (μ_m) and the biomass productivities (P_b) when initial OMW concentrations were increased in the culture medium. In both cases, μ_m and P_b values were increased with the rise in %OMW in the culture medium until 50% of OMW (v/v), then these parameters were rapidly decreased (especially in the case of μ_m) indicating inhibition or toxic effect in the culture media. The highest experimental values of μ_m (0.07 h^{-1}) and P_b (1.25 mg/(L h)) were registered in the culture with 50% of OMW (v/v). After this concentration, these parameters were decreased to 0.0165 h^{-1} and 0.408 mg/(L h) in the culture with 100% of OMW (v/v), in which the lowest values were achieved. This result was expected due to the presence of fat matter, organics acids, pesticide residues and phenolic compounds in the composition of OMW, which are known to harm and inhibit microalgal growth (Hodaifa et al., 2012; Kobayashi and Rittmann, 1982).

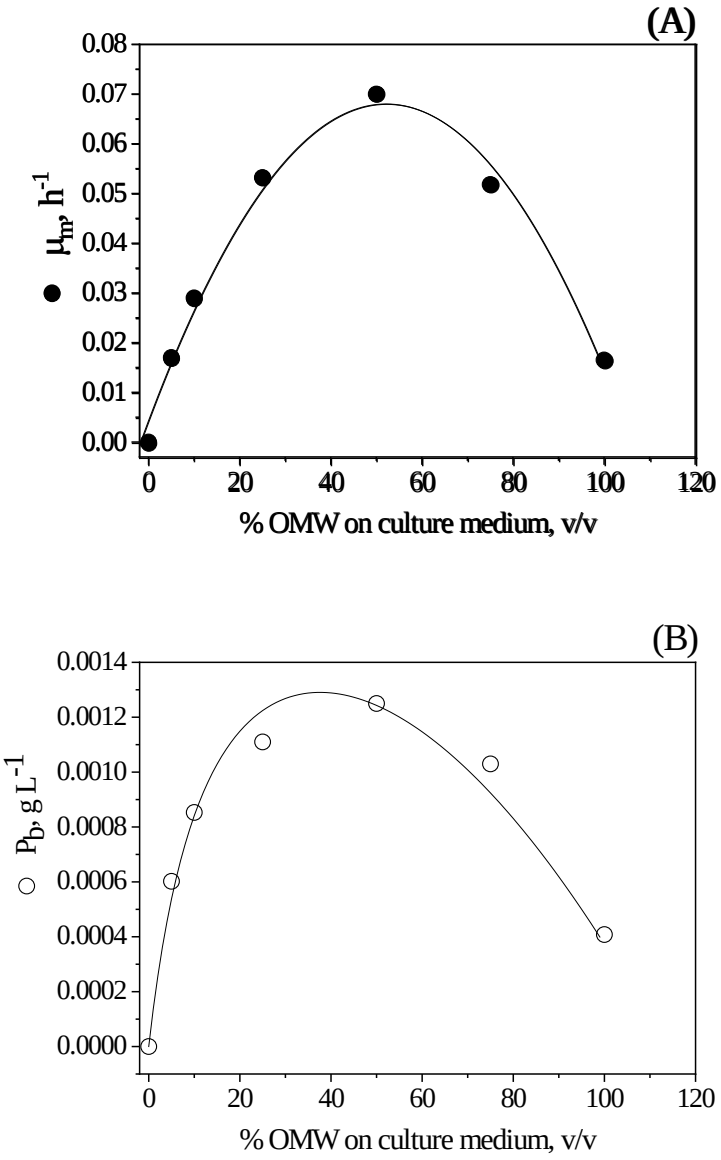

Fig. 3. Variation of maximum specific growth rates (A, black solid line corresponds to model type of Moser, 1985) and volumetric biomass productivities (B, black solid line correspond to the modified Monod model) of *Chlorella pyrenoidosa* culture in different OMW dilutions. Common operational conditions: agitation rate = 200 rpm, T = 25 ºC, aeration rate = 0.5 L/min and continued illumination intensity = 359 $\mu E\, m^{-2}\, s^{-1}$.

After studying various inhibition and toxicity growth models by substrate, the one that best reproduced the experimental variation observed in μ_m with %OMW concentrations was the polynomial model type of Moser (Moser, 1985), Eq. (3),

$$\mu_m = \mu_{m,max} \left(\pm\alpha_o \pm \alpha_1 \%OMW \pm \alpha_2 \%OMW^2\right) \quad (3)$$

where '$\mu_{m,\,max}$ = 0.068 h^{-1}' is the maximum value of the maximum specific growth rate obtained in the different cultures performed and the constant values of 'α_o, α_1 and α_2' are equal to 0.0588, 0.0367 and -3.52×10^{-4}, respectively. The parameters of the goodness of the fit were r^2 = 0.978 and residual sum squares (RSS) = 5.51×10^{-5}. In this sense, it is interesting to indicate that the maximum value for μ_m obtained by the mathematical model is similar to that achieved experimentally (0.07 h^{-1}).

The volumetric biomass productivity was determined by the fit of the x-t data during the deceleration growth phase, as mentioned before. The start of this phase is associated with limited availability of CO_2 (Goldman et al., 1981), light (Evers, 1990) or both, and these two components of the culture were provided at a constant rate. CO_2 was supplied through aeration of the culture medium at 0.5 v/v/min and the incident intensity of illumination was also constant in all experiments and equal to 359 μE m^{-2} s^{-1}. However, due to the colouration of the medium, the attenuation of the light was greater in culture media containing a higher percentage of OMW. This explains the decrease in P_b with the increase of OMW concentration in the culture medium. Just as with μ_m, P_b increases with the rise in OMW in the culture medium until 50% of OMW (v/v), when the maximum biomass productivity, equal to 1.25 mg/(L h), was achieved.

The model that justifies the variation of P_b with the percentage of OMW is the modified Monod model in which the presence of toxic agents or a substance at high enough concentrations were considered (Fig. 3B). This model includes a term of 'K_i %OMW^2' to describe the inhibitory or toxic effect of a nutrient at high concentrations and it is defined by Eq. (4)

$$P_b = \frac{P_{b,max}\ \%OMW}{K_S + \%OMW} - K_i \%OMW^2 \quad (4)$$

where '$P_{b,\,max}$ = 0.002041 g/(L h)' is the apparent maximum value of volumetric biomass productivity without inhibition effect. Though the value of $P_{b\,max}$ is higher, the constant values of K_S = 13.8% and $K_i = 1.42\times10^{-7}$% are consistent with the data obtained experimentally. The parameters of the goodness of the fit were r^2 = 0.961 and residual sum squares (RSS) = 3.22×10^{-8}. P_b values were similar to that registered by Sánchez et al. (2001). In that work, *C. pyrenoidosa* was cultivated in OMW obtained from a continuous olive oil extraction system using 'Decanter' with three exits, this OMW is known as 3-phase system or 'Alpechín' in Spain. However, lower μ_m values (0.011-0.045 h^{-1}) were obtained due to the higher organic matter concentration in OMW from three-phase extraction system (DQO = 40-220 g O_2/L) in comparison with OMW from two-phase extraction system (DQO = 4-16 g O_2/L), (Agabo-García and Hodaifa, 2017).

ii. Biochemical composition of *C. pyrenoidosa* biomass

At the end of the experiments, the harvested biomass of *C. pyrenoidosa* was analysed for proteins, carbohydrates and lipids contents determination. These are the microalgae cells main components. The variation on the biomass composition of *C. pyrenoidosa* for all OMW dilutions is shown in Fig. 4.

Microalgal cells require nitrogen for the synthesis of protein, nucleic acids and phospholipids, and thus the growth of microalgae is believed to be essential for nitrogen removal (Wang et al., 2015). Protein content of the microalgae biomass was increased with the increment of OMW concentration in the culture media (Fig. 4) and ranged from 0.99% (Initial $TN_{culture\ medium}$ = 0.948 mg/L and $\%TN_{final\ \ biomass}$ = 0.155%), in 5% OMW (v/v) culture media, to 51.5% (Initial $TN_{culture\ medium}$ = 17.3 mg/L and $TN_{final\ biomass}$ = 8.25%), in 100% OMW culture medium. It could therefore be concluded that protein content of the microalgae cells was sensitive to changes in nutrient levels. The initial nitrogen content in the low concentration OMW culture medium was not enough for the synthesis of proteins, causing the decrease of the protein content in the biomass at the end of the culture and in the microalgae growth subsequently. Proteins are essential for microalgae growth. Nutrient deficiency could inhibit protein synthesis and microalgae growth subsequently. Zhang et al. (2017) demonstrated the rapid biomass accumulation of *C. pyrenoidosa*

when the microalgae was grown in straw hydrolysate medium and the effectiveness of nitrogen regulation in biomass composition in heterotrophic condition. Hodaifa et al. (2008) obtained similar results with the same OMW and *Scenedesmus obliquus*. In this study, the percentage of protein varied between 6.2% and 30.8%, corresponding to 5% and 50% OMW (v/v) culture media. The biomass protein content of *S. obliquus* reached a value of up to 43.8% (Hodaifa et al., 2013) when the microalgae was cultured in a medium without N deficiency as the Rodríguez-López (Rodríguez-López, 1964) synthetic medium (Becker, 1994).

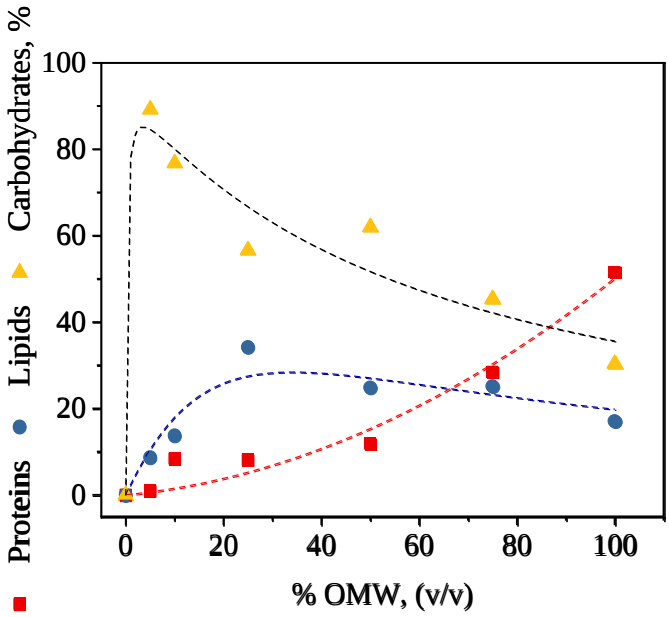

Fig. 4. Variation of biochemical composition of *C. pyrenoidosa* with the variation of the OMW percentages on the culture media.

Carbohydrates content in biomass under low OMW percentages increased because of nutrient deficiency (mainly nitrogen). Under nitrogen stress condition, microalgae store carbohydrates as molecular reserves that can be used as alternative energy sources. This is consistent with previous findings showing that carbohydrate accumulation in microalgae is triggered by nitrogen depletion. On the other hand, cultures with 5% OMW are virtually transparent after primary treatment, which favoured autotrophic culture. In this sense, through photosynthesis

microalgae can convert atmospheric CO_2 along with water and light into organic matter, being carbohydrates the major products. The excess of fixed carbon is commonly stored into carbohydrates, and in stressful conditions, these molecular reserves can be used as alternative energy sources for the production of cell structures (Wang et al., 2015).

In terms of lipids content in *C. pyrenoidosa* biomass, it ranged from 8.71% (5% OMW, v/v) to 34.2% (25% OMW, v/v). In all experiments carried out, the total nitrogen in OMW after primary treatment were varied from 0.489 mg/L (5% OMW, v/v) to 17.3 mg/L (100% OMW, v/v). Nevertheless, the initial TN availability in control synthetic medium of Rodríguez López was = 140 mg/L (Ródriguez-López, 1964). This fact indicated that all experiments in this work were performed under nitrogen stress condition. On the other hand, these results are consistent with those obtained in previous studies in which microalgae were cultivated under stress conditions such as high OMW concentration, nitrogen and phosphate limitation or high salinity. In stress conditions, lipids formation are preferred storage compounds due to its high-reduced state and were packed in cells for the microalgae survival (He et al., 2015; Wang et al., 2015; Yao et al., 2015).

Table 2 shows the identified fatty acids in the algal biomass lipid fraction harvested from the different culture media. Fatty acids were grouped into saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA). In general, higher SFA percentages (85.2%-95.1%) were registered. Moreover, a slightly increase in the SFA percentages was appreciated with the augment of %OMW (more darkness caused by colour effect) in the culture media. The attenuation of light by the gradual change in cultures colour was greater with higher %OMW. Fact that allowed the change of culture behaviour from mixotrophic to heterotrophic. In this sense, Hodaifa et al. (2009) observed for *S. obliquus* biomass that SFA content was higher in the absence of light (heterotrophic growth) than in the presence of light (mixotrophic light-limited cultures). Mixotrophic with high light inhibition and heterotrophic cultures behaved similarly, and the content of SFA approached and even exceeded the heterotrophic value, regardless of the aeration supplied. On the other hand, MUFA and PUFA contents showed the opposite trend, as contents were greater in mixotrophic (low %OMW) than in heterotrophic (high %OMW) cultures (Hodaifa et al., 2009). It is necessary to indicate that the higher percentage of SFA in 5% OMW (v/v) culture is due to the few fatty acids

identified in the lipid fraction of the biomass. This fact could be explained considering the small amount of algal biomass obtained (0.980 mg/L) at the end of the culture.

Table 2. Fatty acid profiles obtained from the lipid fraction of *C. pyrenoidosa* biomass at the end of the experiments.

Fatty acids	Olive-oil mill wastewater concentration, % (v/v)					
	5	10	25	50	75	100
C14:0	n. d	0.80	0.61	0.48	0.56	1.56
C16:1	n. d	n. d	n. d	0.19	n. d	n. d
C16:0	71.9	66.5	74.7	65.7	72.3	63.7
C18:2*n*6	5.43	0.29	0.19	0.31	0.45	0.24
C18:1*n*9	4.66	13.9	4.70	14.3	7.32	8.32
C18:0	10.8	7.11	7.75	8.11	8.60	7.31
C20:0	n. d	2.69	2.91	2.46	1.67	2.49
C22:0	n. d	0.84	0.66	0.40	0.55	1.56
C24:0	n. d	0.62	0.66	0.60	0.67	2.30
C26:0	n. d	4.57	5.20	4.24	5.09	8.36
C28:0	n. d	2.68	2.65	3.24	2.83	4.20
ΣSFA*	82.7	85.8	95.1	85.2	92.3	91.5
ΣMUFA**	4.66	13.9	4.70	14.5	7.32	8.32
ΣPUFA***	5.43	0.29	0.19	0.31	0.45	0.24

**Corresponding to the sum of saturated fatty acids.*
*** Corresponding to the sum of monounsaturated fatty acids.*
**** Corresponding to the sum of poly unsaturated fatty acids.*

The main fatty acids found were palmitic acid (16:0), oleic acid (18:1n9) and stearic acid (18:0). Palmitic acid has been registered the highest percentages (65.7%-74.7%). On the contrary, palmitoleic acid (16:1) was only detected in experiments with 50% (v/v) of OMW. The only polyunsaturated acid identified was 18:2n6 and it was detected in the biomass obtained from all experiments. Higher percentages of linoleic acid (18:2n6) were found in the biomass obtained from low OMW concentration cultures (5% OMW, v/v), but no linolenic (18:3n3), EPA (20:5n3) or DHA were found in any of the experiments. Obtaining a high lipid fraction (34.2% in the culture

with 25% OMW, v/v) in the final biomass gives rise to the possibility of using this fraction for biodiesel production. In this sense, special attention must be paid to the linolenic acid (18:3) and other polyunsaturated fatty acids (≥ 4 double bonds) content of the biomass since the European Standard (EC, 1998) specifies maximum limits of 12.0% and 1%, respectively, for a good biodiesel quality production. All lipid fractions obtained in the experiments are close to that specified by the European Standard (EC, 1998). It is necessary to indicate that higher percentages of saturated fatty acids in the lipid fraction give more stability to the produced biodiesel since these fatty acids are not prone to oxidation.

In any case, the final biomass obtained (0.098143 mg/L-0.143 mg/L) could be used in combination with other substrates for biofuels production or maybe as supplementary substrate in the anaerobic digester for biogas production. In addition, as a last option, it could be used for domestic, commercial or industrial boilers and as a fuel for generators to produce electricity.

4.1.3.5. OMW degradation by microalgae and final treated water quality

Microalgae can consume organic and inorganic nutrients for cell generation. In this work, the biological treatment proposed was based on *C. pyrenoidosa* growth. Fig. 2B shows total carbon species and total nitrogen variation in the global algal culture (OMW+microalgal biomass). A decline in the total organic carbon during the first stages of the culture, corresponding with the exponential growth of *C. pyrenoidosa,* is due to the removal of organic compounds from the culture medium and its conversion into biomass structures. Once the exponential and linear growth phases were finished, the concentration of TC and TOC showed a slight rise explained by the assimilation of smaller quantities of organic compounds due to the cessation of growth and the microalgae death and cell ruptures.

Fig. 2C shows the variation of all carbon species concentration with time in the treated OMW (culture medium) without microalgal biomass. It can be observed a rapid decrease in the starting period, particularly in the first 50 h, corresponding this descent with the exponential growth phase of the microalgae. This result pointed out that total organic matter removal efficiency was dramatically increased during the exponential phase and indicated that the microalgae was able to

assimilate organic compounds as a carbon source through mixotrophic metabolism when both organic carbon and light are present. An increase of the TOC and TC at later stages of cultivation is associated with cell death and ruptures, which leads to an increase in the content of organic compounds in the medium.

In all experiments, IC concentrations (in treated OMW and global culture) were decreased with time (Fig. 2B and C). The reduction of the IC levels during the first 50 h of the culture in parallel with TC and TOC concentrations can be explained by the ability of *C. pyrenoidosa* to grow mixotrophically assimilating organic compounds as carbon sources while using inorganic compounds as electron donors (Chojnacka and Marquez-Rocha, 2004).

After exponential growth, when all the assimilated organic compounds (mainly sugars) were removed, the reduction of IC levels during the last hours of the culture (treated OMW, Fig. 2C) can be explained by the assimilation of inorganic carbon and light by microalgae.

Table 1 shows the treated water characteristics after microalgae growth. In general, for all experiments and characterization parameters, higher removal percentages were registered in cultures in which larger OMW dilutions were used. In this sense, the removal values of %TC, %TOC, %IC and %TN were 74.0%, 75.5%, 71.3% and 87.6%, respectively, in the culture medium formed by 25% of OMW (v/v). These values were decreased to 23.3%, -15.5%, 63.1% and 67.3%, respectively, in the experiment in which undiluted OMW was used. This COD and TOC reduction was observed in the six different culture media, indicating that the microalga was able to use organic carbon and light throughout mixotrophic metabolism. All parameters were decreased throughout the secondary OMW treatment process, with the exception of turbidity and TOC in the culture without OMW dilution, which showed an increase after *C. pyrenoidosa* culture due to the presence of cell debris in the final treated water. In this sense, it is interesting to indicate that after carrying out multiple centrifugations of the supernatant obtained after the first separation by centrifugation of the cell suspension of microalgae, a drop of approximately 30% in the parameters of COD and TOC (data not shown) was observed. The behaviour of removal percentages registered for characterization parameters is consistent with the variation of the maximum specific growth rates and biomass productivities values (Fig. 3).

To determine the effectiveness of the secondary treatment for phenols degradation, their content in the OMW was determined after algal growth. In general, TPCs were decreased through the course of the culture. Furthermore, a steeper decrease can be observed during the exponential phase of growth (Fig. 5A). TPCs removal percentages increased with the augment of %OMW (v/v) in the culture medium. These values were increased from 58.6% to 67.1% in the cultures with 5% and 50% OMW (v/v), respectively, and showed a decrease to 36.4% in the culture constituted by undiluted OMW (Fig. 5B). This behaviour is consistent with the observed variation of the μ_m and P_b values with %OMW in the culture media. In addition, it is interesting to indicate that *C. pyrenoidosa* biomass was able to degrade most of the TCPs (final TPCs < 1 mg/L) present in the culture medium when the initial concentration was below 5.4 mg/L. The highest algal concentration was achieved when initial TPCs content in the culture medium was lower or equal to this value.

Fig. 5B shows the variation of the final %TPCs removal registered in the different culture media. In this sense, many authors have demonstrated the ability of *C. pyrenoidosa* to eliminate high concentrations of phenols and other polluting compounds. Dayana and Bakthavatsalam (2016 and 2017) investigated the degradation effect of *C. pyrenoidosa* (KX686118) on the phenolic effluent of a coal gasification plant. In these previous works, final concentrations of phenols of up to 1.1 g/L were achieved after microalgae growth, registering removal percentages higher than 90%. In addition, Wang et al. (2015) studied triclosan removal and biodegradation in water by using the same microalgae. When *C. pyrenoidosa* was exposed to a series of triclosan concentrations ranging from 100 to 800 ng/mL, more than 50% of triclosan was eliminated by algal uptake from the culture medium during the first 1 h of exposure, reaching the equilibrium after 6 h treatment. In biodegradation experiments, a removal percentage of 77.2% was obtained after the *C. pyrenoidosa* culture in the presence of 800 ng/mL triclosan for 96 h. In addition, Lika and Papadakis (2009) demonstrated that biodegradation of phenolic compounds by microalgae occurs in a shorter time interval during the first stages of cultivation, when all nutrients required by the microalgae are present in the culture medium. When algal cells are grown under constant light intensity and in the presence of organic compounds as carbon source (mainly carbohydrates), there is a substantial increase in the growth resulting in higher biomass, this exponential growth phase corresponds with

the stage when the bioremoval of the phenolic compounds by the microalgae is performed. In this context, it is important to indicate that carbohydrates and phenolic compounds uptake is performed by microalgae. In this sense, Di Caprio et al., (2018) when studying biodegradation of OMW sugars by the green microalga *Scenedesmus sp.* indicated that phenol removal took place immediately after the stop in the consumption of OMW sugars.

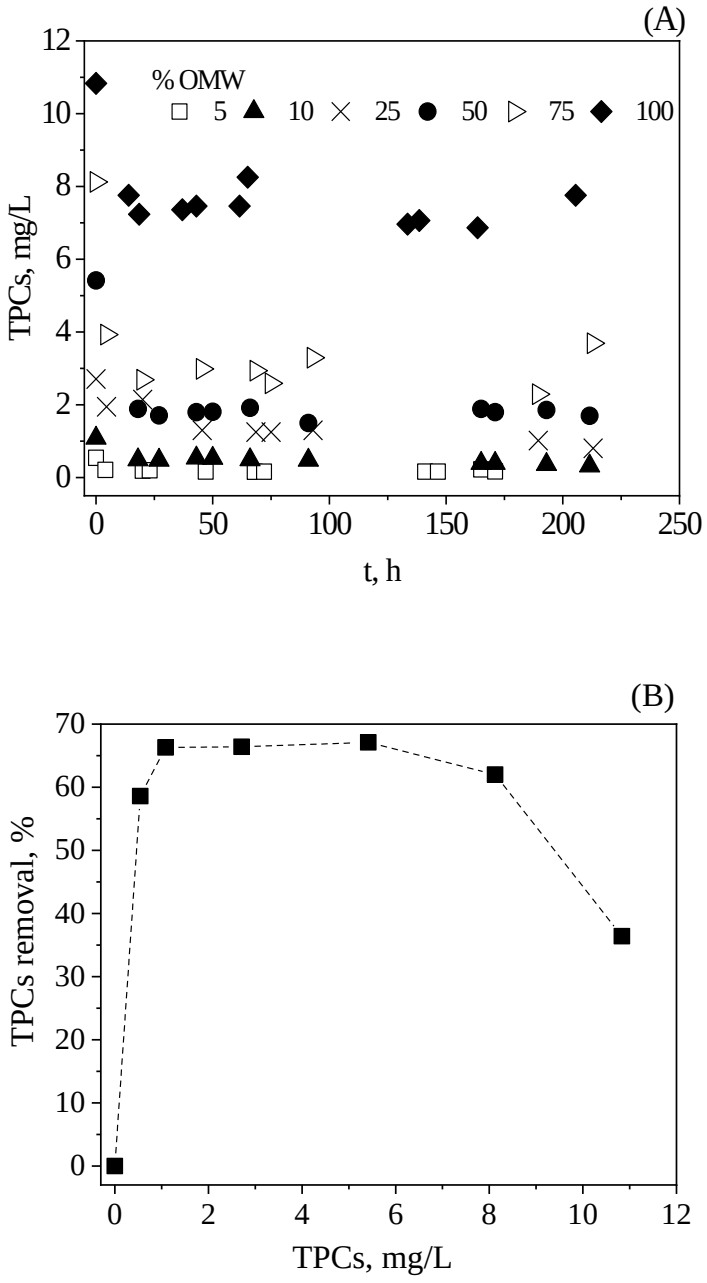

Fig. 5. Variation of total phenolic compounds concentration (A) and final removal percentages of TPCs (B) versus time and TPCs initial concentrations, respectively.

At the end of the process, a high quality treated water was obtained and did not present any toxicity considering that it comes from a combined process where ultraviolet light is applied (which has a disinfecting effect) and microalgae are grown. Parameters registered in Table 1 indicate that treated water could be used for irrigation and discharges to surface water and groundwater or for drinking water.

Spanish environmental standards for treated OMW intended to be used as irrigation water, established that treated water must comply the following parameters pH = 6–9, suspended solids < 500 mg/kg and COD < 1000 mg O_2/L (Resolution of Guadalquivir River Basin president, 2006). In addition, the treated water at the exit of the process comply with European Directive 91/271/EEC where COD < 125 mg O_2/L and TN = 10 mg/L for treated water discharge into receiving waters (European Commission Directive, 1998).

On the other hand, the consolidated text of the Drinking Water Directive with its latest amendments, including Commission Directive (EU) 2015/1787 of 6 October 2015, define that drinking water is all water used in any food-production process undertaking for the manufacture, processing, preservation or marketing of products or substances intended for human consumption unless the competent national authorities are satisfied that the quality of the water cannot affect the wholesomeness of the foodstuff in its finished form. This Directive established chemical parameters and indicator parameters which determined the drinking water quality. These are electric conductivity < 2500 μS/cm, turbidity acceptable to consumers and no abnormal change, TOC = no abnormal change, iron = 0.2 mg/L, sulphate = 250 mg/L, sodium = 200 mg/L and ammonium = 0.5 mg/L. The values obtained for treated OMW from crude OMW concentration < 25% (v/v) have values next to that request by drinking water standards. In any case, if some parameter needs to be adjusted some other units such as ion exchange unit or other membrane technology units could be added.

4.1.4. Conclusion

OMW have a complex composition, which hampers its treatment. The combined process based on physico-chemical and biological treatments is essential for its efficient treatment. The primary treatment (flocculation, photolysis and microfiltration) allowed the elimination of a large part of OMW organic load (96.2% of COD, 80.3% of TOC and 96.6% of TPCs). Secondary treatment eliminated the rest of OMW organic load and the final treated water is suitable to be used for irrigation, discharge to receiving waters or for its reuse in the process itself allowing the closing of water cycle in the factory. The low percentage of sludge generation (mainly during flocculation) can be recirculated to the head of the treatment process or be directly used in composting. After the primary treatment, higher growth rates for *C. pyrenoidosa* (μ_m = 0.07 h^{-1} and P_b = 1.25 mg/(L h)) were registered. Final biomass obtained may be used in direct combustion, methane production or in biodiesel production.

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4.1.5. References

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4.2. COMBINATION OF PHYSICOCHEMICAL OPERATIONS AND ALGAL CULTURE AS A NEW BIOPROCESS FOR OLIVE MILL WASTEWATER TREATMENT

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ABSTRACT

This work presents a new bioprocess design which allows a substantial reduction of organic and inhibitory compounds and a better quality of the final treated water. The process involves a physicochemical (primary) and a biological (microalgae) treatment, which were tested separately with lab equipment, for olive oil mill wastewater (OMW). Primary treatment of OMW involved flocculation-sedimentation by Flocudex CS-51 and microfiltration using a 0.2 μm membrane. Secondary treatment consisted of *Scenedesmus obliquus* culture in different OMW dilutions in ultrapure water as culture media: 5, 10, 25, 50, 75 and 100%. Experiments were performed on a laboratory scale in stirred batch tank reactors. The common operating conditions were: pH = 7, temperature = 25 ºC, agitation rate = 3.33 Hz, aeration rate = 0.5 min^{-1} and illumination intensity = 359 μE m^{-2} s^{-1}. High global removal levels were achieved after primary treatment for chemical oxygen demand (92.6%), total phenolic compounds (98.9%), total organic carbon (75.9%), total nitrogen (63.5%) and inorganic carbon (55.3%). Similar results were obtained for the main OMW constituents after secondary treatment with final harvested biomass rich in energetic compounds, where the highest values of carbohydrates (72.5%) in culture with 5% OMW and lipids (44.9%) in 100% OMW culture were determined.

Keywords: Olive mill wastewater; Flocculation; *Scenedesmus obliquus*; Kinetic growth; Bioprocess.

4.2.1. Introduction

Microalgae can be considered as the microorganisms of the future due to their potential in numerous applications. By way of example, they are sustainable bioremediation agents and a source of energy, proteins, natural pigments, etc. In addition to its use in cosmetics, pharmaceutical applications, human and animal feed, aquaculture, etc. (Mata et al., 2010).

Microalgae are promising microorganisms characterized by its easy culture, high growth rate and biomass productivity. In addition, microalgae can grow in simple conditions with solar light and inorganic nutrients. The use of synthetic media for microalgae cultivation at industrial scale is economically unviable due to the high costs of chemicals. This fact implies the need to seek cheaper alternatives to form culture media. In this sense, the use of waste and its transformation into by-products for the microalgae cultivation is a good alternative (Hu et al., 2017; Mata et al., 2010).

Generally, wastewaters have macro, micro and trace nutrients that can be used by microalgae. Double goals can be achieved: wastewater treatment and generation of biomass with high economic value. In brief, it is a sustainable and eco-friendly bioprocess (Hu et al., 2017). Species such as *Ankistrodesmus falcatus*, *Botryococcus terriblis*, *Chlorella pyrenoidosa*, *Scenedesmus obliquus* or *Spirulina platensis* have shown an efficient growth and high removal rates of contaminants (heavy metals, pesticides, etc.) contained in many wastewaters as urban and those generated by industries such as aquaculture, soybean processing, dairy industries, etc. (Wang et al., 2016).

Industrial wastewaters are heterogeneous and complex since they contain suspended solids, chemicals, greases, etc., which can lead to growth inhibition. In this sense, the correct design of the bioprocess is key to achieve the highest removal of organic and inorganic load from wastewater. At the same time, a proper bioprocess design allows a more rapid microorganism growth and higher biomass production (Komolafe et al., 2014; Mohd Udaiyappan et al., 2017).

In conventional wastewater treatment, different stages are generally applied. Primary treatment is intended to eliminate large solids and particles. Secondary seeks to the bioremediation of organic compounds through the action of microorganisms. In addition, in some countries, a tertiary treatment is applied to reuse the final treated water (Mohd Udaiyappan et al., 2017). Olive

mill wastewaters (OMW) are one of the most polluting within the agro-food industry waste, constituting a major concern in the Mediterranean area, where 30×10^6 m^3 of OMW are generated per year. Press, batch, and continuous methods are used for olive oil extraction. Nowadays, continuous methods (two and three-phases) are used in most of the producing countries. In both cases different wastewater biochemical composition is obtained (Hodaifa et al., 2013; Ioannou-Ttofa et al., 2017). In general, OMW has a dark brown colour, unpleasant odour, low pH, high turbidity, organic load, polysaccharides, sugars, proteins and phenolic compounds such as hydroxytyrosol, tyrosol, p-hydroxyphenyl acetic acid, p-coumaric acid and caffeic acid, etc. (Amor et al., 2015; García and Hodaifa, 2017; Yalili Kiliç et al., 2013). Phenolic compounds (e.g. > 5 mg/L become toxic for *Chlorella pyrenoidosa*) are responsible for the phytotoxic effect and antibacterial activity of OMW, which causes eutrophication, pollution of soils and water resources (Malvis et al., 2019). Currently, OMW storage in evaporation ponds is the most common practice for its management. This system, based on the water removal by evaporation, does not provide a solution for the remaining solid phase. Additionally, it leads to the contamination of water resources and the generation of bad odours (Ioannou-Ttofa et al., 2017). Another alternative proposed and used in some countries is the direct spread on agricultural lands. However, not all countries have this option in its legislation due to the great impact of OMW on soils properties such as pH, electric conductivity, nitrogen and phosphorous availability, etc. (Mechri et al., 2007; Mekki et al., 2006). For this reason, several researchers have proposed physicochemical (sedimentation, flocculation, etc.) (Hodaifa et al., 2015), biological (aerobic activated sludge) (Alrawashdeh and Al-Essa, 2019), anaerobic digestion (Gnaoui et al., 2020), composting (Hachicha et al., 2009), membrane filtration (micro-, ultra- and nanofiltration) (Paraskeva et al., 2007) and chemical oxidation methods Fenton (Nieto et al., 2011), Photo-Fenton (García and Hodaifa, 2017), ozonisation (Siorou et al., 2015), TiO_2 photocatalysis (Hodaifa et al., 2019), etc.). In this sense, Paraskeva et al. (2007) combined natural sedimentation, ultrafiltration, nanofiltration and reverse osmosis and recuperated the solid fraction, the phytotoxic fraction with high molecular weight, water for fertilization (nutrient fraction) and a second concentrated phytotoxic fraction with the potential to be used as growth inhibitors of some native plants, respectively. Markou et al. (2012) obtained a microalgae biomass (*Spirulina platensis*) rich in carbohydrates and proteins after OMW pretreatment with sodium

hypochlorite. Malvis et al. (2019) combined flocculation, photolysis and microfiltration with algal culture (*Chlorella pyrenoidosa*) for OMW treatment and generation of microalgae biomass rich in energetic compounds.

This research aims to study the ability of *Scenedesmus obliquus* to use two-phases OMW as a substrate by reusing its nutrients. In this sense, two goals are achieved: OMW bioremediation and valuable biomass generation. Primary and secondary treatments are designed to accomplish these purposes. Primary consists of flocculation-sedimentation unit to eliminate solids, turbidity and part of OMW colour, followed by microfiltration unit with 0.2 μm membrane to remove organic colloidal matter. Secondary treatment consists of microalgal cultures (5, 10, 25, 50, 75 and 100% of OMW/water). Then, kinetic parameters such as specific growth rates and volumetric biomass productivities were determined. Final biomass value was evaluated through the biochemical composition. Furthermore, the treated water quality during and at the end of the process was determined.

4.2.2. Materials and Methods

4.2.2.1. Microorganism and photobioreactor

The microorganism used in this work was the freshwater green microalga *Scenedesmus obliquus* CCAP 276/3A. Stock cultures were maintained in solid Rodríguez-López Medium (Rodríguez-López, 1964) solidified with agar. Then, cultures were maintained at room temperature and continuous artificial illumination.

Experiments were performed in sterile conditions, at laboratory scale, in stirred batch tank reactors with 1 L work volume and 10 cm (diameter)×16 cm (high) dimensions. All material and glass bioreactors were sterilized in an autoclave at 121±1 ºC for 30 min. Culture media were sterilized by membrane filtration using a membrane of cellulose nitrate with 0.2 μm (pore size).

4.2.2.2. Culture media

OMW was taken from a reservoir of an olive oil mill with continuous centrifugation process using a decanter with two outlets (olive oil and pomace). The olive oil extraction plant was in Seville (Spain). The flocculation-sedimentation was performed during 90 min in a 1 L Imhoff cone using a commercial flocculant (Flocudex CS-51). Optimal flocculant has been chosen at 100 mg/L according to a previous study of Hodaifa et al. (2015). The mixture of flocculant with OMW was carried out in two stages. First, high stirring rate at 11.7 Hz (700 rpm) was applied for 1 min to perform fast and uniform mixing of flocculant with the OMW. Second, slow stirring rate at 5.83 Hz (350 rpm) during 30 min was performed to allow the formation of flocs and increase their size.

Flocculated OMW (F-OMW) was used to form the culture media (F-OMW/Ultrapure water) at different concentrations 5, 10, 25, 50, 75 and 100%. Microfiltration through a 0.2 μm membrane was used for the removal of colloidal particles and culture media sterilization. The pH of culture media was adjusted to an initial value of 7.0 with 0.1 mol/dm^3 NaOH and 0.1 mol/dm^3 HCl solutions.

The common culture conditions used were: temperature = 25ºC, aeration rate = 0.5 min^{-1}, pH value = 7.0, agitation rate = 3.33 Hz (200 rpm) and artificial continuous white light with illumination intensity = 359 μE m^{-2} s^{-1}. A cell suspension from sterile Rodríguez-López Medium (Rodríguez-López, 1964) was used as initial inoculum for OMW cultures at 0.00405±0.00236 g/L.

4.2.2.3. Physicochemical characterization of raw industrial olive mill wastewater

The high complex composition of OMW hampers its treatment (Dermeche et al., 2013). The main physicochemical characteristics of raw OMW used in this work are summarized in Table 1. The parameters turbidity = 714 FTU, chemical oxygen demand (COD) = 5839 mg/L, total phenolic compounds (TPCs) = 322 mg/L, total organic carbon (TOC) = 328 mg/L and total nitrogen (TN) = 58.9 mg/L, represent the organic matter, the main parameter to consider from the environmental point of view. High concentration of carbon and nitrogen is desirable since both are required nutrients for microalgae growth. Raw OMW presents approximately half the concentration

(2.4 times) of total nitrogen than the mineral synthetic medium of Rodríguez-López (Rodríguez-López, 1964), with 140 mg/L, which is normally used as control medium for the same microalgae (Órpez et al., 2009). TPCs were transferred to the industrial raw OMW during olives crushing and olive oil washing (García and Hodaifa, 2017).

In addition, OMW also contains inorganic salts measured as inorganic carbon (IC) = 318 mg/L and orthophosphate (PO_4^{-3}) = 43.1 mg/L. Phosphorous concentration in raw OMW is notably lower than that of Rodríguez-López, with phosphorous = 160 mg/L (Hodaifa et al., 2009). The presence of orthophosphate is highly relevant in metabolism phosphorylation reactions (Fazal et al., 2018).

Chloride has been shown to be toxic for microalgae growth at high concentrations. In this sense, Figler et al. (2019) proved for *S. obliquus* cultured in Bold's Basal medium, that 5.8 g/L of NaCl (3.51 g/L of Cl-) caused 50% growth inhibition (EC_{50}) after 4 days, and concentrations higher than 10 g/L of NaCl (6.1 g/L of Cl^-) were toxic. In addition, according to Li et al. (2013), this value for *Chlorella pyrenoidosa* ranged from 19.7 g/L to 36.3 g/L. The chlorides concentration in raw OMW used in this work is only 204 mg/L and 98.5 mg/L, after primary treatment, at the beginning of *S. obliquus* cultures, so the growth of *S. obliquus* is adapted/inhibited at this low concentration.

In addition, sulphur, a required component of some amino acids, vitamins and sulfolipids, was detected at high concentration in the form of sulphate (320 mg/L). Iron (1.19 mg/L) is necessary for photosynthesis, due to its role in enzymatic reactions in photosystem I and II. Furthermore, it is a key factor in the synthesis of essential proteins such as ferredoxin and cytochrome (Cao et al., 2014; Fazal et al., 2018). Several studies have shown the effect of iron concentration on the biomass and lipid content in different microalgal species. Liu et al. (2008) proved that increasing the iron concentration in the medium caused an increase in the content of biomass and lipids in Chlorella vulgaris. Additionally, Abd El Baky et al. (2012), got a lipid content increase in *Scenedesmus obliquus* from 5.6% to 28% by increasing the iron concentration in the culture medium.

Table 1. Characterization of raw and treated OMW during treatment process.

Parameter	Raw OMW	Primary treatment		Secondary treatment
		Flocculated	Microfiltration	*S. obliquus*
pH	6.25±0.8*	Natural**	Natural	8.9±0.1
Conductivity, mS/cm	1.97±0.5	1.30±0.2	1.44±0.2	6.8±0.1
Turbidity, FTU	714±6.0	53.5±2.1	4.09±1	25.6±0.6
COD, mg O_2/L	5839±60	2484±11	433±10	192±5
TPCs, mg/L	322±3.0	4.2±0.1	3.62±0.2	2.33±0.2
TC, mg/L	646±27	561±11	222±7	148±6
TOC, mg/L	328±2.0	530±8.0	79.2±6	62.9±7
TN, mg/L	58.9±3.6	27.8±0.7	21.5±1	5.99±0.6
IC, mg/L	318±4.0	31.3±1.3	142.3±2	85.1±0.4
Iron, mg/L	1.19±0.01	1.10±0.1	0.67±0.01	0.72±0.02
Chloride	204±4.0	116±4	98.5±1.3	156±6
Sulphate, mg/L	320±30	84.8±2.9	53.8±1.1	56.8±0.3
Sodium, mg/L	0.943±0.1	0.782±0.02	0.05±0.005	0.99±0.12
Orthophosphate, mg/L	43.1±2.1	21.7±1.3	21.3±2	9.24±0.46

**Standard deviation value.*

***pH without modification.*

4.2.2.4. Analytical methods

The following parameters were determined for raw and treated OMW: pH value, electric conductivity (EC), turbidity, chemical oxygen demand (COD), total phenolic compounds (TPCs), total carbon (TC), total organic carbon (TOC), total nitrogen (TN), inorganic carbon (IC), total iron, chloride, sulphate, sodium and orthophosphate.

pH, electric conductivity (EC) and turbidity values were directly measured by using a pH-meter Crison, mod. GLP 22C, Conductimeter Crison, mod. GLP31 and Turbidimeter Hanna, mod. HI93703, respectively.

Chemical oxygen demand was determined photometrically at 620 nm according to German standard methods (DIN 38409 H41).

The determination of total phenolic compounds was performed by making it react with a derivative thiazol, giving a purple azo dye, determined photometrically at 475 nm according to the standard methods (DIN 38402 A51; ISO 8466-1).

Total carbon, total organic carbon, inorganic carbon and total nitrogen concentrations were determined using a Total Carbon and Nitrogen Analyzer provided by Skalar Company, mod. Formacs^{HT} and Formacs^{TN}.

Total iron ions determination was performed through the reduction of all iron ions to iron (II) ions in a thioglycolate medium with a derivative of triazine. This reaction results in a reddish-purple complex that was photometrically determined at 565 nm according to the standard methods (DIN 38402 A51; ISO 8466-1)

Sulphates and orthophosphates were determined photometrically at 420 nm and 690 nm, respectively, according to the standard methods (DIN 38402 A51; ISO 8466-1 1990).

Sodium content was directly determined by using a selective ion electrode for each ion (Crison, mod. GLP 22C).

Furthermore, biomass generated and biomass biochemical composition were determined. For biomass concentration (x, g/L), a volume of 5 ml of microalga suspension was taken and centrifuged at 50 Hz (3000 rpm) for 10 min. The obtained biomass pellet was washed three times with ultrapure water and measured at 600 nm in a UV-visible Spectrophotometer. A linear calibration curve between absorbance and dry biomass was established. In this sense, a linear relationship from the experimental data of dry weight-cell concentration (g/L) versus absorbance was obtained. The experimental data were determined from samples taken during and at the end of all *S. obliquus* cultures.

Total pigments (chlorophyll a, chlorophyll b and carotenoids) were determined by a photocolourimetric method after its extraction with 90% acetone as described by Ritchie (2008). The total chlorophylls and total carotenoids contents were calculated according to the equations described by Jeffrey and Humphrey (1975) and by Strickland and Parsons (1972), respectively.

At the end of each culture, biomass was separated and dried at 105 ºC. Then, total lipids, proteins and fatty acids content were determined.

The total lipid content of the biomass was extracted by a micro-soxhlet extractor using a n-hexane as solvent for 24 h.

Fatty acids (FA) identification and quantification was performed according to Lepage and Roy (1984) in a gas chromatograph (Hewlett-Packard, Model 5890 Series II) equipped with a flame ionization detector through its transesterification into fatty acid methyl esters (FAME).

The crude protein content was calculated after the determination of total nitrogen concentration by a total carbon and nitrogen analyser provided by Skalar Company (mod. FormacsHT and FormacsTN) according to the formula provided by Becker (1994), %Crude proteins = %TN × 6.25.

The total carbohydrate content was calculated by considering that proteins, carbohydrates, lipids, pigments and genetic materials (considered approximately about 1%, Becker, 1994), are the main components of algal biomass.

4.2.2.5. Statistical methods applied

To confirm the reproducibility of the experimental data reported, the cultures were made at least in duplicate and the analytical methods were applied at least in triplicate. In the duplicated experiments, biomass growth was monitored, and the final wastewater quality was determined. Graphics and statistical methods used were available in OriginPro 8.0.

4.2.3. Results and Discussion

4.2.3.1. Bioprocess designed for *Scenedesmus obliquus* growth

The complex composition of olive mill wastewater, the high organic load and the presence of compounds that inhibit the growth of microorganisms are the main factors that limit the application of conventional technologies (mainly biological treatments) on its treatment. In addition, this kind of treatments generate large quantities of sludge that must be managed, reduced, or eliminated. In fact, up to now, there is not a solution for this wastewater and it is only managed in large accumulation reservoirs for its evaporation during the summer months. Not to mention that proposed methods such as direct ozonisation, forced evaporation, etc. have a higher cost (Lee et al., 2019; Tsintavi et al., 2013).

This work proposes the use of microalgae for olive mill wastewater treatment since it does not imply the generation of a sludge at the end of the process. In addition, the generated algal biomass has a high economic value since it can be used for biofuels production in a substantial way, without forgetting the ability of microalgae to eliminate atmospheric carbon dioxide, contributing to the reduction of the greenhouse effect.

In order to decrease the organic matter content (precisely, COD and turbidity), including inhibitor growth compounds (phenolic compounds) in the wastewater, it is necessary its treatment before being used in algal cultures. In this sense, Floculdex CS-51, a cationic polyelectrolyte (organic polymer for food use) with high molecular weight, soluble in water and based in polyacrylamide, was used based on its great capacity to remove organic matter and phenolic compounds (Hodaifa et al., 2015). On the other hand, to work under sterile conditions, microfiltration with 0.2 μm

membrane was chosen to eliminate microorganisms (fungus, yeasts and bacteria), reduce turbidity and improve light penetration.

For real OMW, a bioprocess involving a physicochemical as primary and a biological as secondary treatment (tested separately with lab equipment) was designed. The physicochemical treatment consisted of flocculation plus microfiltration units. Biological treatment was based on *S. obliquus* growth in different dilutions of industrial OMW as culture media. For this proposed process in its approach, it was considered the operational ease in its execution and operation. Low operational costs were achieved due to the natural sedimentation-flocculation without the addition of chemical compounds, only a small concentration of low-price flocculant was used. In addition, this process includes the production of algal biomass, which is not usually included in other conventional treatment processes.

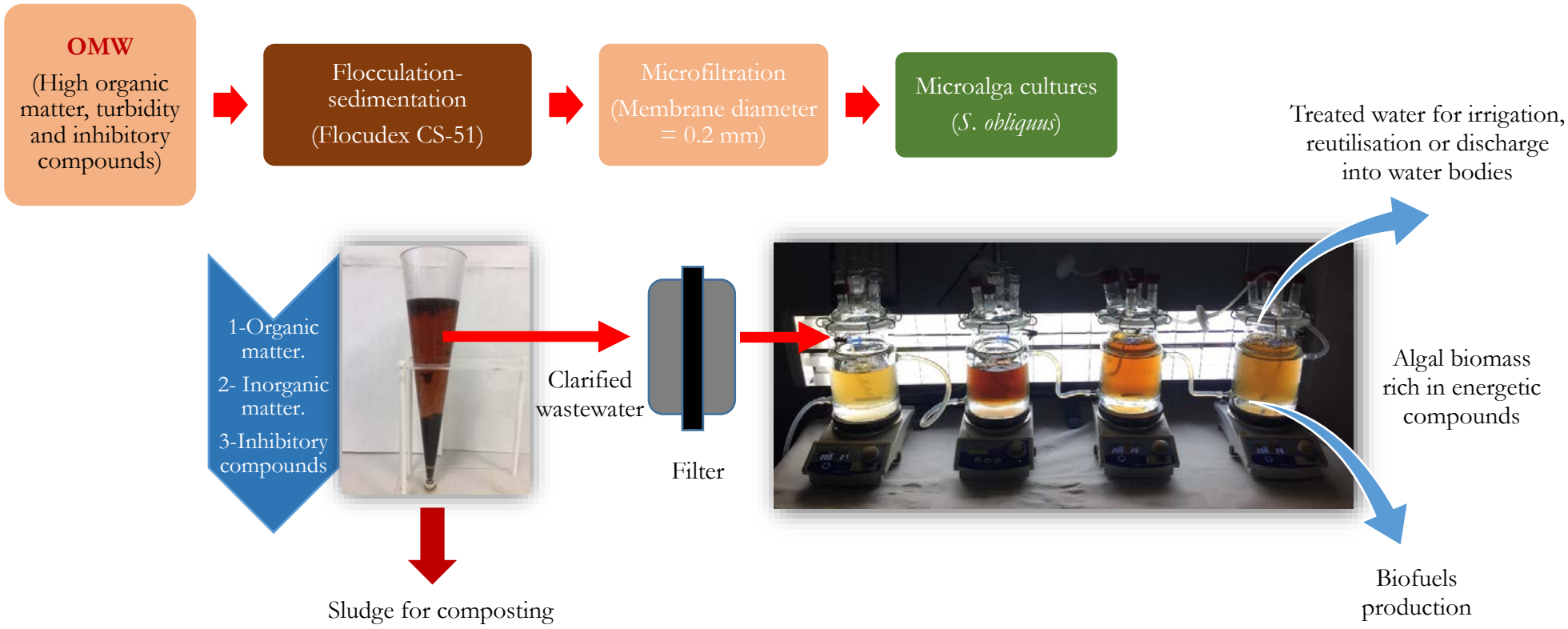

Fig. 1. Schematic representation of the proposed bioprocess for OMW treatment

i. Effect of primary treatment on wastewater characteristics

OMW composition before and after flocculation and microfiltration was determined with the aim to establish the nutrient removal by each operation.

In primary treatment total solids were notably reduced, resulting in the decrease of inhibitory compounds, turbidity and colour. In this sense, high reduction rates were achieved in the main parameters studied (Table 1).

Through flocculation, results showed that conductivity, turbidity, IC, COD, TPCs, TN and orthophosphate were reduced by 34%, 92.5%, 90.2%, 57.5%, 98.7%, 52.8% and 49.7%, respectively. The aim of this stage was to separate and reduce the total solids and total suspended solids content, determined in terms of turbidity. Despite TOC concentration which was increased from 328 mg/L to 530 mg/L. This fact may be due to the flocculant residue in treated OMW. In the same way, a decrease in the concentration of iron (7.56%), chloride (43.1%), sulphate (73.5%) and sodium (17.1%) was also determined.

In microfiltration unit, the following reduction percentages were registered: 98%, 82.6%, 13.8%, 85.1%, 22.7%, 39.1%, 15.1%, 36.6%, 93.6% and 1.84% for turbidity, COD, TPCs, TOC, TN, iron, chloride, sulphate, sodium, and orthophosphate, respectively.

The primary treatment proved to be effective in the reduction of most wastewater parameters. Flocculation could be highlighted as the most effective stage in terms of some of the most harmful compounds for microalgae growth, such as phenols and chloride. The presence of phenols in the culture medium results in inhibition for microalgal growth and smaller cell size (Duan et al., 2017). The establishment of a primary treatment based on flocculation and microfiltration in the new proposed bioprocess is essential due to the role of flocculation in the removal of turbidity and OMW discoloration, allowing a greater light penetration in the culture. Microfiltration allowed higher removal rates of organic matter and iron, which at high concentrations can inhibit *S. obliquus* growth.

ii. Secondary treatment based on *Scenedesmus obliquus* culture

Fig. 2A shows the variation of the biomass concentration through the experiment time for the 75% OMW culture. In all experiments with OMW ≥ 50%, a higher adaptation of *S. obliquus* to the culture media was observed by showing an abrupt increase (Lag phase, Fig. 2A) in the biomass concentration during the first 3 h of culture. This fact may be due to the higher availability of one or more essential nutrients.

In the exponential growth phase microalgae have a balanced growth due to the available nutrients in the culture medium and thus, cells divide at a constant rate depending upon the culture media composition and operating conditions, which results in biomass accumulation. The duration of this phase ranged from 19 h (25% OMW) to 72 h (100% OMW). The longest exponential phase in 100% OMW medium is due to the higher availability of essential nutrients at higher OMW concentrations.

The maximum specific growth rate, μ_m, was determined during the exponential growth phase according to equation (1),

$$ln\left(\frac{x}{x_o}\right) = \mu_m\, t + a \qquad (1)$$

where 'x, g/L' is the biomass concentration at any time of the experiment, 'x_o, g/L' is the biomass concentration at the beginning of the experiment (t = 0 h), 'μ_m, h^{-1}' is the slope of the line and corresponds to the maximum specific growth rate, 't, h' is the time and 'a' is the intercept.

Fig. 2B shows that μ_m values were increased at lower OMW concentrations (μ_m = 0.035 h^{-1} in 5% OMW) and decreased (μ_m = 0.0232 h^{-1} in 100% OMW) when the OMW concentration in the culture media was ≥ 50%. This behaviour may be due to the presence of inhibitory compounds (as residual oil) or light limitation by the increase of culture colour with the augment of OMW concentration in the culture media.

After studying various inhibition and toxicity growth models by substrate, two of them reproduced the experimental variation of μ_m with %OMW concentrations. The first corresponds to the mathematical model of Teissier (1936), Eq. (2),

$$\mu_m = \mu_{m,max} \left[e^{-S_0/K_I} - e^{-S_0/K_s}\right] \quad (2)$$

where '$\mu_{m,\ max}$ = 0,036 h^{-1}' is the maximum theoretical value determined for the maximum specific growth rate obtained, S0 is the percentage of OMW in culture media, K_I = 193% is the value of the inhibition constant and K_S = 1.39% is the value of the slope for ½ $\mu_{m,\ max}$. The parameters of the goodness of the fit were r^2 = 0.964 and residual sum squares (RSS) = 2,46×10-5.

The second model corresponds to the mathematical model proposed by Hodaifa et al. (2008), Eq. (3),

$$\mu_m = \frac{\mu_{m1} K_s S_0 + \mu_{m2} S_0^2 + \mu_{m3} K_i K_s}{K_i K_s - K_i S_0 + S_0^2} \quad (3)$$

where S_0 is the percentage of OMW, μ_{m1} = 0.04 h^{-1} would correspond to the previously described $\mu_{m,\ max}$, μ_{m2} = 0.0223 is a constant value for μm at the highest %OMW (100% OMW), μ_{m3} = 1,086 × 10^{-6} is a constant value for μm in the absence of OMW in the culture medium at S_0 = 0, K_S = 2.56% and K_I = 7.77%, which is the value at which the inhibition appears. The parameters of the goodness of the fit were r^2 = 0.996 and RSS = 3.96×10^{-6}.

In view of the results, it can be concluded that the $\mu_{m,\ max}$ = 0.036 h^{-1} obtained in the Teissier model (1936) is lower than that obtained by Hodaifa et al. (2008), μ_{m1} = 0,04 h^{-1}, since this value corresponds to the theoretical value without inhibition. The optimal value of μ_m was determined when %OMW was equal to 7.77% and 7.07% for Hodaifa et al. (2008) and Teissier model (1936), respectively. However, Hodaifa et al. (2008) is the model that best fits the experimental behaviour since K_I = 7.77% is consistent with that observed experimentally in contrast to the value determined by Teissier model (1936), (K_I = 193%).

In all experiments, a deceleration growth phase with linear behaviour was observed (Fig. 2A). In this phase of growth, the volumetric biomass productivity was calculated according to Eq. (4),

$$x = P_b\, t + b \tag{4}$$

where 'P_b, mg/(L h)' is the line slope and corresponds to the value of volumetric biomass productivity and 'b' is the intercept.

Fig. 2C shows the P_b values tendency. Data were fit to a second-degree polynomial model ($r^2 = 0.985$). The maximum value registered was $P_b = 0.896$ mg/(L h) in culture with 100 % OMW medium.

The appearance of this linear phase may be related to limited availability of CO_2 (Goldman et al., 1981), light (Evers, 1990) or both, and these two components were provided at a constant rate to the culture media. CO_2 was supplied through the aeration of the culture medium at constant value equal to 0.5 min^{-1} and the incident light intensity supplied to the photoreactors surfaces was the same for all experiments and equal to 359 $\mu E\ m^{-2}s^{-1}$. In this sense, nitrogen is an essential nutrient and it varied among the cultures due to the OMW dilution. Nitrogen is essential in proteins, chlorophyll, DNA, etc., formation. Low nitrogen concentrations inhibited *S. obliquus* division, leading to decreasing microalgal biomass productivity. TN content in 5% OMW culture medium was equal to 1.44 mg/L in comparison with 21.5 mg/L in 100% OMW medium. This variation in the culture media presented limited availability of nitrogen. In addition, the duration of the linear phase ranged from 27.5 h (5% OMW) to 240 h (50% OMW), then it decreased to 168 h in the culture with 100% OMW. This behaviour is consistent with the hypothesis of nitrogen limitation. The decrease in the phase duration in cultures with OMW concentrations higher than 50% may be due to the light limitation caused by the increase in culture coloration. Several microalgal species such as *Chlorella vulgaris*, *Chlamydomonas reinhardtii* or *Scenedesmus subspicatus* have shown similar behaviour under nitrogen limitation conditions (Dean et al., 2010; Ikaran et al., 2015). *C. vulgaris* showed prolonged growth under N-replete conditions and yielded 1.8 times higher final biomass in comparison with N-limitation conditions (Ikaran et al., 2015). Similarly, *C. reinhardtii* and *S.*

subspicatus exhibited restricted cell division when cultured at low N concentrations; among three nitrogen concentration conditions (high-N culture = 19.6 mg/L, intermediate-N culture = 3.0 mg/L and low-N culture = 0.8 mg/L), both strains showed the lowest biomass in the low-N medium and notably increased biomass generation under high N-conditions (Dean et al., 2010).

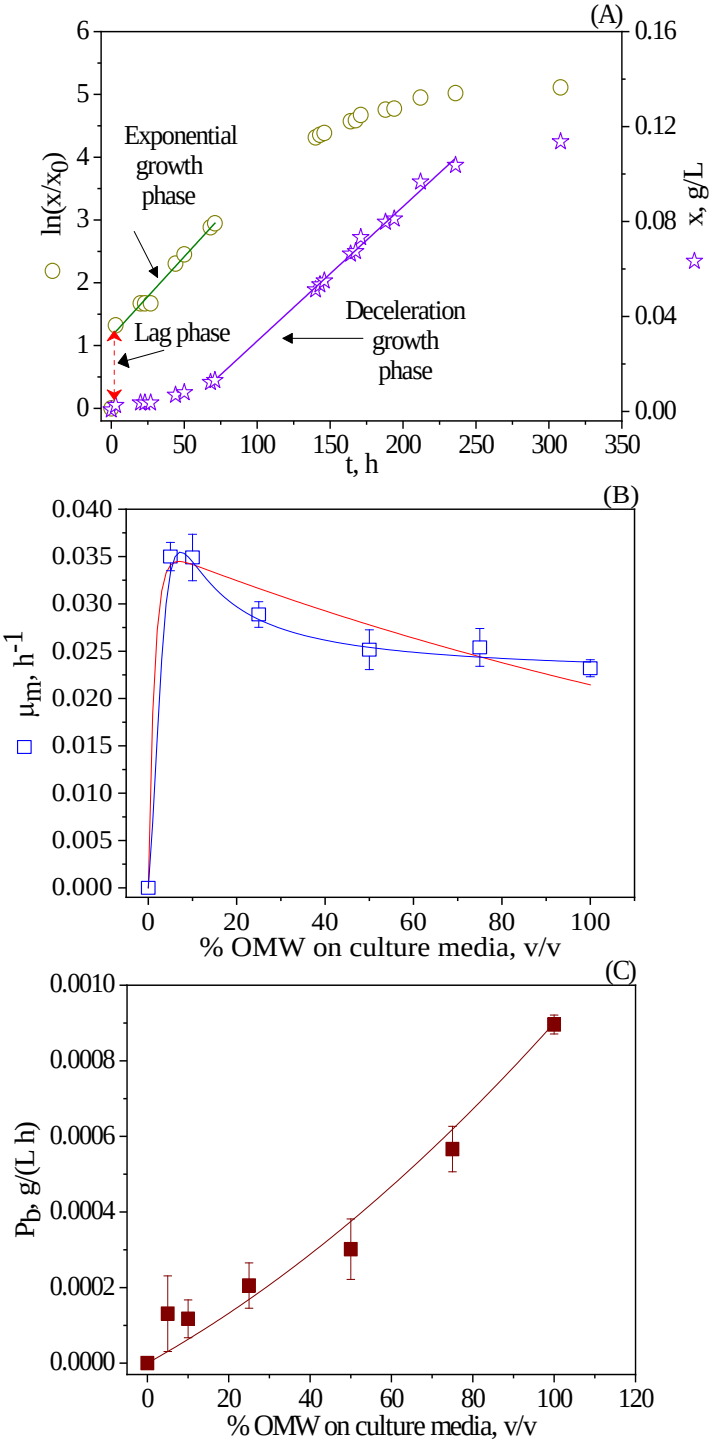

Fig. 2. A) *Scenedesmus obliquus* growth curves on 75% OMW. B) Maximum specific growth rates (μ_m) variation versus different OMW dilutions as culture media (Red and blue solid lines correspond to Teissier model (1936) and Hodaifa et al. (2008), respectively). C) Volumetric biomass productivities (P_b) variation versus different OMW dilutions as culture media (— Solid line corresponds to simple second order equation model). Common operational conditions: agitation rate = 3.33 Hz, T = 25 ºC, aeration rate = 0.5 min^{-1} and continued illumination intensity = 359 μE m^{-2} s^{-1}. Error bars represent standard deviation.

4.2.3.2. Culture medium effect on final biomass generation and its biochemical composition

The final biomass concentration at the end of the cultures ranged from 0.029 g/L (5% OMW) to 0.21 g/L (100% OMW). Although these concentrations are low, the main goal of this work is the OMW treatment and in parallel, microalgal biomass with added value is generated. Today, urban wastewater is treated and citizens pay the cost of this treatment within our drinking water bill. No operations units included in this bioprocess are expensive. In fact, in our upcoming research works, the microfiltration unit is removed from the bioprocess and this is performed in non-sterile conditions.

A ternary diagram was plotted (Fig. 3) to represent biomass biochemical composition (lipids, proteins and carbohydrates, the main microalgae cells components). In this diagram, it can be clearly observed that lower nitrogen concentration in culture media resulted in higher carbohydrate content (72.5% and 18.7% in 5% and 50% of OMW, respectively). Then, carbohydrate and lipid contents increased to 43.2% and 44.9% in culture with 100% OMW, respectively (Fig. 3). Microalgae can accumulate carbon into energy-rich compounds (carbohydrates and lipids) as a response of a growth stress (Ho et al., 2012). These results could be therefore due to light limitation caused by the light attenuation because of medium coloration, which is greater with the increasing of %OMW and thus the expected variation (Markou et al., 2012).

In addition, this fact was confirmed by the influence of turbidity in the light reaching microalgae inside the bioreactor, since the turbidity values in input to microalgae after dilution were varied as following 1.22 FTU, 1.53 FTU, 1.89 FTU, 2.43 FTU, 3.40 FTU and 4.09 FTU for cultures with 5%, 10%, 25%, 50%, 75% and 100% of OMW, respectively.

Protein content showed the opposite trend to that observed for carbohydrates and lipids. The increase in nitrogen concentration (1 mg/L to 10 mg/L) implied a protein content augment (from 4.65% to 64.2%). Then, protein content decreased to 10.8% in the culture with 100% OMW (Fig. 3). This reduction may be due to the nutrient limitation as a result of an oil layer on the cells surface which blocked nutrients access, since higher OMW percentage in culture media implies high residual olive oil in the culture medium (Hodaifa et al., 2008). Nitrogen and phosphorous are

essential constituents in protein structure and its synthesis is also related to both nutrients in the culture media.

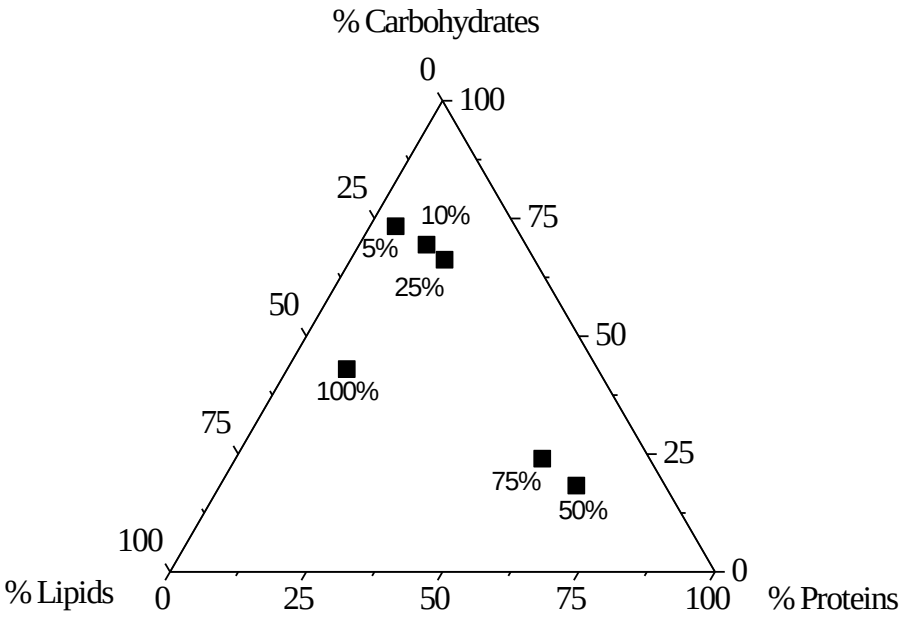

Fig. 3. Biochemical composition (percentages in dry weight of lipids, carbohydrates and proteins) of *S. obliquus* represented as ternary plot illustration for all cultures studied (5, 10, 25, 50, 75 and 100% OMW). Common operational conditions: agitation rate = 3.33 Hz, T = 25 ºC, aeration rate = 0.5 min^{-1} and continued illumination intensity = 359 μE m^{-2} s^{-1}.

Table 2 shows the fatty acids contents determined in the lipid fractions of algal biomass. These fatty acids are grouped into saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA). Fatty acids concentration is influenced by operating parameters as light intensity, nutrient availability, pH and temperature (Miró-Casas et al., 2003). In the experiments, the last two parameters were kept constant and thus the variability in fatty acid profiles could be attributed to nutrients availability and light intensity. In this sense, the following fatty acid percentages were registered: saturated (51.1%-64.1%), monounsaturated (22.6%-37.5%), polyunsaturated (0.17%-

0.18%) and the sum of saturated plus monounsaturated (86.8%-98.9%). Among the saturated fatty acids, the most abundant was palmitic acid (42.3%-54.8% of C16:0), followed by stearic acid (6.18%-7.10% of C18:0) and among the monounsaturated, the most abundant was oleic acid (21.4%-35.1% of C18:1n9). The high saturated and monounsaturated fatty acid percentages obtained (86.8% and 98.9%, respectively) are the most suitable components for high quality biodiesel production since they contribute to some important properties of biodiesel as density, viscosity, oxidative stability and heating value (Feng et al., 2014). The only polyunsaturated fatty acid identified was linoleic (C18:2n6) at low concentrations (< 1 %) in the biomass obtained from culture media ≤ 25% OMW. High polyunsaturated fatty acids levels are not desired for biodiesel production due to their ease degradation and oxidation (Ge et al., 2018).

The coefficient of variation (CV) revealed that among all fatty acids, the highest variation was obtained for C16:1 (104.6%), C24:0 (100.9%), C28:0 (40.6%), C20:0 (37%), C14:0 (20.8%), C26:0 (17.1%), C16:0 (8.2%) and C18:0 (4.6%), since %CV values were higher than 2%. Regarding the calculated sums, significant variations were obtained for the unidentified (57.3%), monounsaturated (15.4), saturated (7.6%) and saturated plus monounsaturated (4.5%) fatty acids.

In general, the cultures with 10% to 75% of OMW did not register a significant difference in the saturated fatty acid percentages (63.4±0.85%). The difference determined in cultures with 5% and 100% may be due to the high unidentified fatty acids (8.23% and 11.4%).

The harvested biomass could have direct use in combustion or by its fractionation into lipids, carbohydrates and inert fractions. The first fraction could be destined to biodiesel production. The second in alcoholic production through anaerobic fermentation and the third could be used in anaerobic digesters for biogas production. All these possibilities allow the generation of energy, which could be transformed into different forms such as heat, fuel, and electricity. Although this biomass has nutritional value, the current legislation does not allow its use in human or animal feeding. In any case, the biomass represents a sustainable resource for energy production and a clean energy. In brief, this is an added value in form of energy alongside the wastewater treatment (main objective of this bioprocess).

Table 2. Fatty acid profiles obtained on lipid fraction of *S. obliquus* biomass harvested at the end of the experiments.

% Fatty acids	Olive-oil mill wastewater concentration, %						CV*, %
	5	10	25	50	75	100	
C14:0	0.42	0.6	0.42	0.37	0.37	0.33	20.8
C16:1	2.15	1.24	11.4	2.15	1.26	2.4	104.6
C16:0	48.9	54.8	52.5	53.8	50.7	42.3	8.2
C18:2*n*6	0.17	0.18	nd	nd	nd	nd	2.9
C18:1*n*9	30.7	21.4	25.4	28.1	31.6	35.1	15.4
C18:0	6.49	6.23	6.62	6.18	7.10	6.56	4.6
C20:0	1.22	1.58	1.37	2.14	2.26	0.59	37.0
C22:0	0.4	0.51	0.46	0.41	0.41	0.37	10.7
C24:0	0.23	0.33	0.30	0.25	1.57	0.22	100.9
C26:0	0.14	0.17	0.22	nd	nd	0.16	17.1
C28:0	0.76	nd	0.17	0.97	0.79	0.63	40.6
ΣSFA**	58.7	64.2	62.1	64.1	63.2	51.1	7.6
ΣMUFA***	32.9	22.6	36.8	30.2	32.9	37.5	15.4
ΣPUFA****	0.17	0.18	nd	nd	nd	nd	2.9
ΣSFA+ ΣMUFA	91.6	86.8	98.9	94.3	96.1	88.6	4.5
Unidentified	8.23	13.0	1.10	5.70	3.90	11.4	57.3

**Coefficient variation = standard deviation*100/mean.*
***Corresponding to the sum of saturated fatty acids.*
**** Corresponding to the sum of monounsaturated fatty acids.*
***** Corresponding to the sum of polyunsaturated fatty acids.*

4.2.3.3. Pollutants removal by *S. obliquus*

Microalgae can consume organic and inorganic nutrients from wastewaters for cell generation. This removal can be calculated by measuring the following parameters: TC, TOC, IC, TN, PO_4^{-3} and total iron ions.

i. Total organic and inorganic carbon removal

Fig. 4 (A and B) shows the variation of TOC and IC concentrations in OMW (without *S. obliquus* biomass) over the course of the experiments. For both concentrations of carbon species, a sharp decline in these values was observed during the first 27 h of the cultures, except in the case of 5% OMW culture. This descent matches with the exponential growth phase in which the maximum specific growth velocity was determined. In the subsequent growth phases, a slightly decrease in these values was observed. In the case of OMW without dilution (100% OMW), an increase in final TOC and IC values was registered due to the release of intracellular compounds from ruptures of dead cells (Malvis et al., 2019; Martínez, 2000).

TOC (-23.5%, 43.5%, 39.3%, 67.4%, 48.5% and 20.5%) and IC (73.2%, 76.8%, 88.5%, 95.8%, 50.1% and 40.2%) removal percentages were determined for 5%, 10%, 25%, 50%, 75% and 100% OMW cultures, respectively. The negative percentage indicates an increase in the final TOC values for 5% OMW culture.

The maximum removal values for TOC and IC were registered in the culture with 50% of OMW. However, the maximum specific growth velocities were determined in the cultures with 5% and 10% of OMW. These good results are due to the lack of toxic constituents at low OMW concentrations by dilution effect. In addition, this fact could be explained by considering that *S. obliquus* changed its metabolism from autotrophic (in uncoloured culture with 5% of OMW with virtually no TOC uptake (Fig. 4A)) to mixotrophic growth (in the cultures with OMW concentration among 10% and 50%) to heterotrophic cultures for the other cultures (75% and 100% OMW). The augment of OMW in the culture media increases cultures colour. Similar results were previously showed in our work, demonstrating that high fat matter and colour in undiluted OMW act as limiting factors for *S. obliquus* growth and nutrients uptake (Hodaifa et al., 2012).

ii. Total nitrogen removal

Total nitrogen of OMW (without *S. obliquus*) variation throughout the experiments is shown in Fig. 4C. It can be observed, in all experiments, a steeper decrease during the first hours of cultivation, which corresponds with *S. obliquus* exponential growth phase. Then, nitrogen uptake

slightly decreased and remained virtually constant at the end of the culture. Global total nitrogen reduction was equal to 54.8%, 76.8%, 77.5%, 98.2%, 98.2% and 72.1% for culture media with 5%, 10%, 25%, 50%, 75% and 100% OMW, respectively. These removal percentages are consistent with protein concentration determined in final biomass generated. Highest protein contents 64.2% and 55.4% were achieved in cultures media with 50% and 75% of OMW, respectively. Lower nitrogen availability (1.44 mg/L) in 5% of OMW culture resulted in minor biomass and protein generation 0.029 g/L and 4.65%, respectively. In this sense, nitrogen disposal must be controlled since excess nitrogen lead to eutrophication water bodies (García and Hodaifa, 2017).

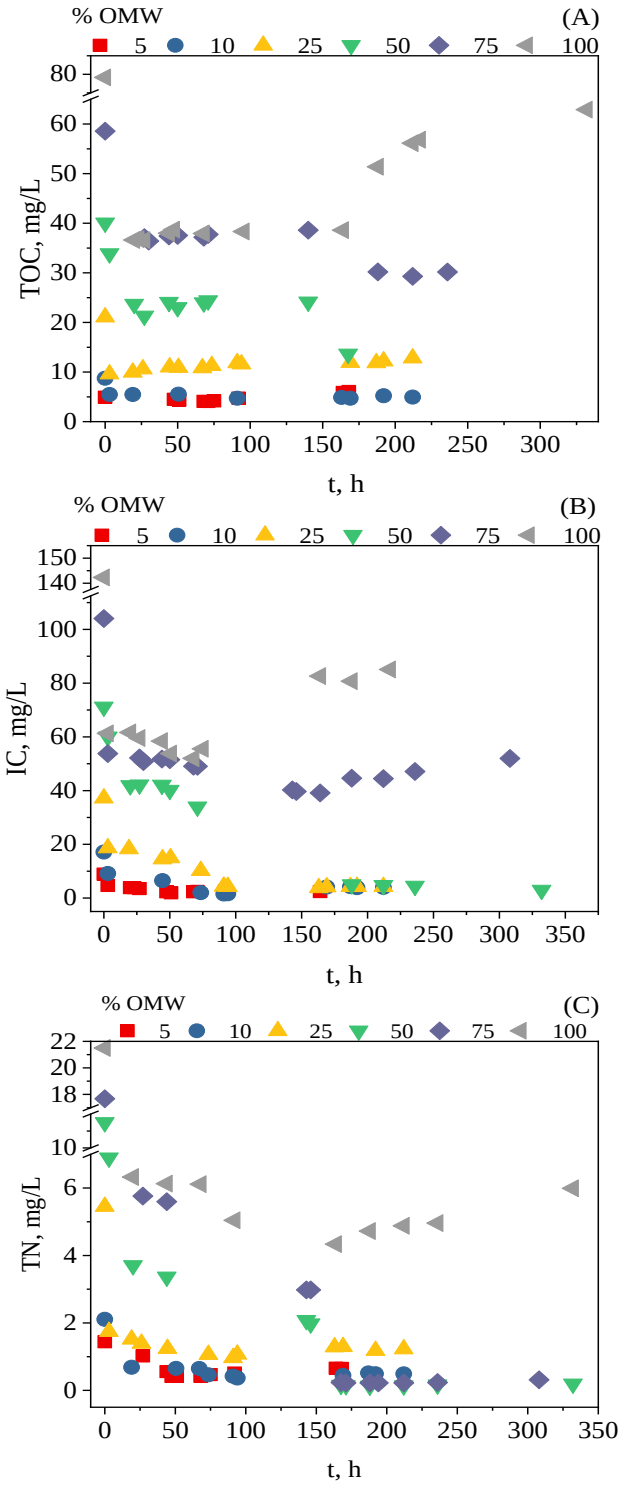

Fig. 4. Variation of total organic carbon, TOC (A), inorganic carbon, IC (B) and total nitrogen, TN (C) on the treated OMW dilutions (without algal biomass) along the cultures. Common operational conditions: agitation rate = 3.33 Hz, T = 25 ºC, aeration rate = 0.5 min^{-1} and continued illumination intensity = 359 µE m^{-2} s^{-1}. The experimental data of TOC, IC and TN were determined at least twice with coefficient variation (CV) < 2 (Coefficient variation = standard deviation*100/mean).

iii. Total phenolic compounds removal

Fig. 5A shows the variation of TPCs concentration in OMW over the course of the experiments. In the 100% OMW culture, the TPCs removal was performed in two steps. In a first step, a pronounced decrease during approximately the first 50 hours of the culture was observed. Then, a slow decrease with linear behaviour. On the other cultures, it can be considered a linear behaviour (zero-order equation model) for TPCs concentration throughout the experimental time, since the initial TPCs concentrations in these cultures (5% to 75% of OMW) were less than 3 mg/L. In this way, TPCs final concentrations below 1 mg/L were achieved in culture media containing ≤ 25% OMW. In any case, it is important to point out that only small consumption of phenolic compounds was expected since phenolic compounds are toxic for microalgae.

Fig. 5B shows TPCs removal rates and final global removal percentages obtained in the different culture media studied. The highest removal TPCs rate values (-0.00106 and -0.00160 mg/(L h)) and elimination percentages (54.4% and 59.1%) were obtained in cultures with 5% and 10% of OMW. Cultures with OMW percentages equal or higher than 25% registered similar removal percentages around 35%. The removal percentages of TPCs tendency shows an inhibition effect of phenolic compounds at higher OMW concentrations.

Several studies have shown the ability of different microalgae strains to remove phenols from wastewaters. Cheng et al. (2017) proved that the oleaginous microalgae *Tribonema minus* was able to efficiently degrade phenols from an initial concentration in the culture media of up to 700 mg/L and this TPCs biodegradation was directly influenced by the initial concentration of TPCs in the medium. In this work, the maximum phenol removal percentage was equal to 94.6% at an initial phenol concentration of 250 mg/L. Lee et al. (2015) indicated that *Spirulina maxima* is able to grow on synthetic wastewater culture media with phenols up to 400 mg/L, achieving a 97.5% of phenol removal. Furthermore, Stephen and Ayalur (2017) obtained high phenols removal levels (91%) when growing *Chlorella pyrenoidosa* on a phenolic effluent of a coal gasification plant (20% of effluent). In this study, the phenolic compounds in the culture media were varied from 282 mg/L to 846 mg/L.

Finally, according to APHA (Hussain et al., 2015) all treated OMW could be directly discharged into public sewers, with a permissible limit of phenols equal to 5 mg/L. However, cultures with 5%, 10%, 25% and 50% are suitable for discharge into inland surface waters, with an admissible limit of 1 mg/L. In general, all treated OMW could be discharged into inland surfaces waters and public sewers since the final TPCs concentration are remarkably close to the lowest value required.

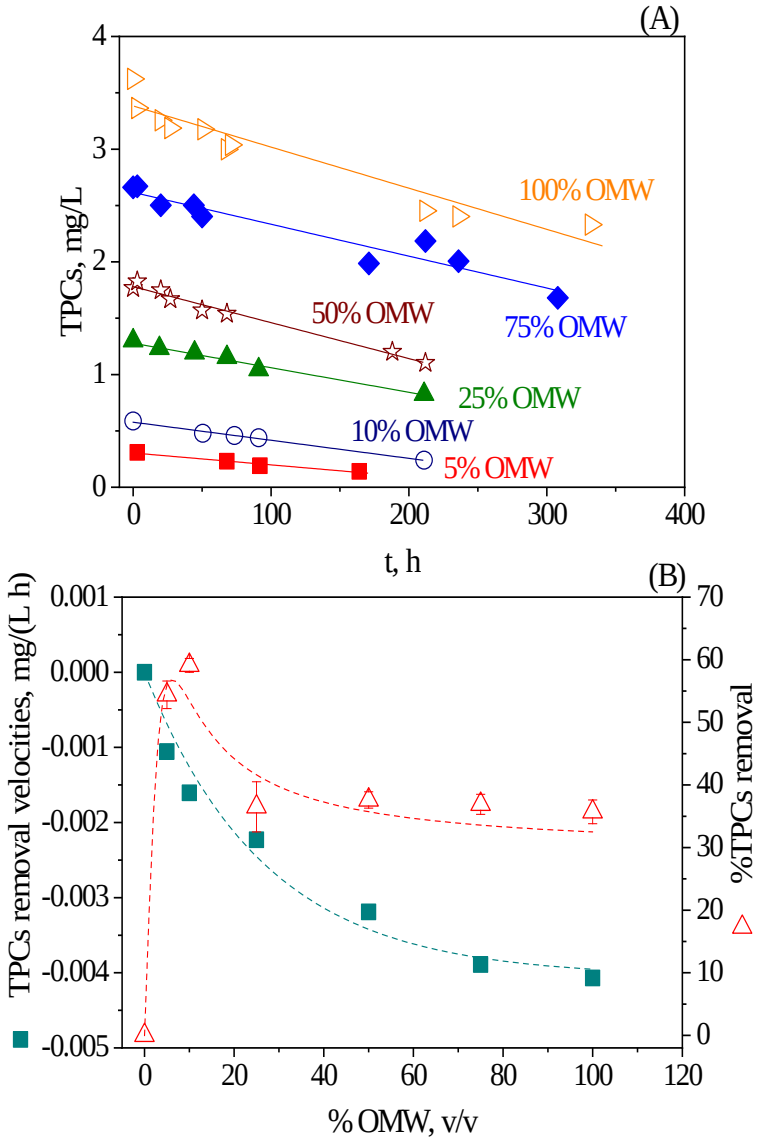

Fig. 5. A) Variation of total phenolic compounds (TPCs) concentration in OMW along the cultures. B) Total phenolic compounds removal velocities and final TPCs removal percentages. Common operational conditions: agitation rate = 3.33 Hz, T = 25 ºC, aeration rate = 0.5 min^{-1} and continued illumination intensity = 359 μE m^{-2} s^{-1}. Error bars represent standard deviation.

iv. Reduction on minority compounds

Orthophosphate and total iron as minor compounds were measured at the beginning and the end of the experiments, since orthophosphate have a key function in the synthesis of proteins, nucleic acids and phospholipids and iron is a crucial element in photosynthesis and respiratory transport chains of electrons. The orthophosphate removal percentages in OMW were ranged from 53.7% to 70.2% in cultures with 75% and 5% of OMW, respectively.

Iron removal percentages ranged from 5.91% to 46.1% in cultures with 75% and 25% of OMW. The consumption of this element by *S. obliquus* is due to that iron improves the photosynthetic activity and increases the biomass productivity (Liu et al., 2008).

4.2.4. Conclusions

The combination of a physicochemical treatment (primary treatment) based on flocculation and microfiltration plus microalgal growth of *S. obliquus* culture (secondary treatment) has been established for the treatment of industrial OMW. This combined process allowed the wastewaters treatment and the generation of a valuable microalgae biomass. Primary treatment allowed high global removal levels of organic and inorganic matter, which resulted in a culture media with less turbidity, colour and colloidal particles, favouring culture illumination. As a result of the previous treatment, algal growth registered maximum specific growth rate (μ_m = 0.035 h^{-1}) and biomass productivity (P_b = 0.896 mg/(L h)) in cultures with 5% and 100% of OMW, respectively. In addition, high removal percentages up to 67.4% (50% OMW), 95.8% (50% OMW), 98.2% (50% OMW) and 59.1% (10% OMW) were determined for TOC, IC, TN and TPCs, respectively. On the other hand, the final biomass obtained was rich in energetic compounds, with maximum carbohydrate and lipid contents up to 72.5% (5% OMW) and 44.9% (100% OMW), respectively.

The scale up of the industrial OMW treatment could be established as a combination of physicochemical (flocculation and microfiltration) and microalgal treatments (*S. obliquus* culture). For biodiesel production, the best operating conditions to apply are: OMW without dilution, aeration rate 0.5 min^{-1}, agitation speed 3.33 Hz, continuous illumination, and temperature equal to

25 ºC. In these conditions, highest biomass (0.21 g/L) and lipids (44.9%) generation were obtained. From the point of view of pollutants removal, the use of a culture medium with 50% of OMW resulted in the following removal percentages: TOC 67.4%, IC 95.8%, and TN 98.2%. For phenolic compounds removal, the highest removal velocities (-0.00106 and -0.00160 mg/(L h)) and percentages (54.4% and 59.1%) were determined in the culture media with 5% and 10% of OMW, respectively. In any case, in a real process, temperature and illumination would be variables imposed by natural conditions, which means that solar light and ambient temperature would be used.

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4.2.5. References

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4.3. CULTIVATION OF *SCENEDESMUS OBLIQUUS* IN MIXTURES OF URBAN AND OLIVE-OIL MILL WASTEWATERS FOR THE DUAL APPLICATION OF ALGAL BIOMASS PRODUCTION AND WASTEWATER TREATMENT

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ABSTRACT

Olive-oil mill wastewater (OMW) is a great environmental problem because of its high organic load plus another antioxidant compounds as phenolic compounds. On the other hand, the treated urban wastewater (UW) in depuration plants, which have primary, secondary and, in some cases, tertiary treatment processes, is directly disposed to public waterways. Both wastewaters could be used as sources for microalgal culture media constitution. These wastewaters are rich in nitrogen and phosphorus compounds such as ammonium, nitrates and phosphates as well as organic and inorganic compounds. In this work, the revalorization of these wastewaters throughout the microalgal biomass production and the reutilization of the final treated water has been studied. The crude OMW was pretreated by flocculation and ultraviolet light before microalgal culture. All microalgal experiments were performed in batch photo-bioreactors (1 L work capacity) at laboratory scale. The operational conditions were: agitation rate = 200 rpm, T = 25 ºC, aeration rate = 0.5 L min^{-1} and continuous light with illumination intensity equal to 359 μE m^{-2} s^{-1}. Results revealed that the new proposed process lead to the improvement of the final water quality. High removal percentages of organic matter and nitrogen species were registered. The final biomass obtained was characterized by high energetic compounds percentages (carbohydrate and lipid contents).

Keywords: microalgae; *Scenedesmus obliquus;* olive-oil mill wastewater; urban wastewater; lipids.

4.3.1. Introduction

One of the major concerns that industries must face is the large amount of wastewater that are generated because of their activity. In addition to industrial effluents, huge quantities of urban wastewaters (UW) are generated by industrialized countries (Órpez et al., 2009). This substantial volume of residual waters must be treated to avoid environmental contamination and to ensure public health with safe water supplies (Mohd Udaiyappan et al., 2017). In addition, according to the World Health Organization (WHO), freshwater scarcity is a matter that will affect > 40% of the world's population in the next 50 years (WHO, 2006). To solve these problems, new methods for wastewater treatment must be explored in order to get suitable water for reuse in irrigation, discharge to receiving waters or for being reused in the same industries where they are generated (Gutiérrez-Alfaro et al., 2018).

Between the different treatment processes for residual waters, bio-treatment with microalgae is particularly attractive since microalgae are photosynthetic microorganisms which convert solar energy into useful biomass and incorporate nutrients such as nitrogen or phosphorus from the effluents (Abdel-Raouf et al., 2012). In addition, microalgae present many other advantages such as ease of cultivation since they can grow almost anywhere with little attention using unsuitable water for human consumption (Suganya et al., 2016). Its use for wastewater treatment requires the proper selection of the microalgae specie with a series of specific characteristics such as high growth rate, high lipid content and productivity and a large tolerance to polluting compounds such as metal ions, pathogenic microorganisms or phenolic compounds among many other components which can harm microalgae growth and are extensively present in different wastewater streams (Wang et al., 2016).

Wastewaters can be classified in several categories such as municipal, pharmaceutical, agro-industrial or textile dyes wastewater among many others (Wang et al., 2016). Each type has its own physicochemical characteristics as well as its own nutrient composition and presence of potential inhibitors (Hodaifa et al., 2013; La Scalia et al., 2017). These effluents require a treatment before being dumped into rivers, lakes or the sea, in order to achieve environmentally safe levels of the

contaminants present in their composition (ammonium, nitrates, phosphates, etc.), which can contribute to the eutrophication of the receiving effluents (Órpez et al., 2009).

Urban wastewaters (UW) are generated as a combination of water and wastes from homes, commercial and industrial facilities. UW are characterized by containing high concentrations of toxic compounds, organic matter, pathogenic microorganisms etc. (Hodaifa et al., 2013). On the other hand, olive-oil mill wastewater (OMW) is a secondary product generated during the olive oil extraction process characterized by its dark brown color, strong odor, acid pH as well as high values for the most polluting parameters: biological and chemical oxygen demand (BOD and COD, respectively), phenolic compounds, nitrogenous compounds (La Scalia et al., 2017) as well as tannins, pectins, lignins, fatty acids etc. (Dermeche et al., 2013).

In this work, the use of UW as well as mixtures with OMW as culture medium for *Scenedesmus obliquus* was studied. The proposed process consisted of a primary treatment, based on a physicochemical treatment, followed by a biological treatment performed by the microalgae. The primary treatment was applied to raw OMW and consisted of flocculation-sedimentation and photolysis by artificial UV light. In all cases a real raw OMW and UW were used. To achieve the aim of this work, physicochemical characteristics of both wastewaters, microalgal biomass production and its biochemical composition were determined. From the experimental results obtained, the kinetic growth parameters were calculated. Final treated water quality and its reuse were established.

4.3.2. Experimental

4.3.2.1. Microorganism and culture conditions

The freshwater microalgae used was *Scenedesmus obliquus* CCAP 276/3A which was supplied by the Culture Center for Algae and Protozoa, Oban (UK). Experiments were carried out in sterile conditions, on a laboratory scale in stirred batch tank reactors with illumination on frontal side and the following characteristics: working capacity = 1 L, diameter = 10 cm and height = 16 cm.

4.3.2.2. Experimental procedure

Urban wastewater was obtained from a plant located in Seville (Spain). Samples were taken from the tertiary treatment. Olive oil mill wastewater was obtained from an olive oil extraction plant from the same province in which olive oil is extracted by the two-phase centrifuge process.

Mixtures of OMW with UW as well as single UW, previously filtered and sterilized through a membrane with 0.2 μm pore size, were used as culture media. Prior to the preparation of the mixtures, the flocculation-sedimentation and photolysis of the raw OMW was performed.

The flocculation-sedimentation had a duration of 90 min. An Imhoff funnel and the commercial flocculant Flocudex CS-51 were used in this stage (concentration = 100 mg/L). The photolysis was performed in a batch stirred photoreactor with total capacity equal to 750 cm^3 (work volume = 600 cm^3). A commercial medium pressure UV immersion lamp, model TQ 150 Brand HNG Germany G4, 150 Nº 5600 1725 (Standard) was used.

For the preparation of the culture media, the following concentrations of OMW were added to raw UW: 0%, 5% and 10% (v/v). The common culture conditions were: temperature = 25ºC, pH = 7, aeration rate = 0.5 L min^{-1}, pH value = 7, magnetic agitation speed = 200 rpm and continuous light with illumination intensity equal to 359 μE m^{-2} s^{-1}.

S.obliquus inoculum consisted of a preculture of cells grown in Rodríguez-López (Rodríguez-López, 1964) mineral medium solidified with agar at 2% (v/v) and incubated for seven days under continuous illumination at room temperature. In each experiment, cells were transferred to the sterilized culture medium after resuspension in sterilized ultrapure water.

4.3.2.3. Analytical methods

i. Biomass concentration monitoring

Biomass concentration was determined through the measurement of the absorbance of the cell suspension in ultrapure water at 600 nm.

ii. Wastewater characterization

The characterization of crude and treated wastewaters was performed through the determination of the following parameters: pH value, electric conductivity, turbidity, chemical oxygen demand (COD), total phenolic compounds (TPCs), total organic carbon (TOC), total carbon (TC), inorganic carbon (IC), total nitrogen (TN), nitrate-nitrite (NN), chloride (Cl^-), sulphates (SO_4^{-2}) and total iron (Fe), according to Hodaifa et al. (2015). Ammonium (NH_4^+), potassium (K^+) and sodium (Na^+) were determined by Crison selective electrode, mod. GLP 22. Orthophosphate (PO_4^{-3}) was measured by the Macherey-Nagel test (0.2-5 mg/L).

iii. Lipids, carbohydrates and proteins determination

Biomass obtained at the end of the culture was separated by centrifugation at 3000 rpm for 5 min and washed three times with distilled water. After drying at 105 ºC, total lipids, proteins and fatty-acids contents were determined.

Total lipids were extracted in a micro-soxhlet extractor using n-hexane as solvent. Fatty acids profiles were determined and identified by gas chromatography (Hewlett–Packard, Model 5890 Series II equipped with a FID detector). The crude protein content was calculated after the determination of total nitrogen concentration using a Total Carbon and Nitrogen Analyzer provided by Skalar Company, mod. Formacs^{HT} and Formacs^{TN} according to the following equation: %Crude proteins = %TN×6.25.

The total carbohydrate content was obtained by considering that algal biomass is formed by proteins, carbohydrates, lipids, pigments and genetic material (considered approximately about 1%).

4.3.3. Results

4.3.3.1. The wastewaters

Table 1 shows the physical and chemical parameters of the different wastewaters used as *S. obliquus* culture media. The raw OMW was also characterized before flocculation and UV photolysis

as follows: conductivity = 1.9 mS/cm, turbidity = 714 FTU, COD = 5839 mg O_2/L, TPCs = 322 mg/L, TC = 1400 mg/L, TOC = 646 mg/L, IC = 318 mg/L, TN = 58.9 mg/L, NH_4^+ = 4.44 mg/L, SO_4^{-2} = 1276 mg/L, PO_4^{-3} = 43.1 mg/L, Na^+ = 0.94 mg/L and Fe = 1.19 mg/L. In this sense, for the use of wastewaters as culture media for microalgae, it must contain a proper nutrient profile, being carbon, nitrogen and phosphorous sources the most essential components for microalgal biomass generation.

With respect to the OMW treated by flocculation and artificial UV light it must be highlighted its high organic load, determined in terms of turbidity = 32.9 FTU, COD = 3746 mg O_2/L, TPCs = 21 mg/L and TOC = 372 mg/L. Nevertheless, TN = 6.56 mg/L indicates a nitrogen deficiency in OMW. The presence of ortho-phosphate (PO_4^{-3} = 26.8 mg/L) in the culture media plays an important role in microalgae cell growth and metabolism through phosphorylation reactions (Fazal et al., 2018). High chloride (Cl^- = 580 mg/L) and sulphate (SO_4^{-2} = 320 mg/L) concentrations were detected. These two last compounds can harm microalgae growth since they are highly inhibitory to microalgal growth. High iron concentration is not desired, the low concentration detected in raw OMW can be explained by the use of drinking water in food industries for washing raw materials. All these organic and inorganic nutrients can be used by microalgae to generate biomass (Fazal et al., 2018).

With respect to raw UW it must be highlighted the high presence of chloride (Cl^- = 202 mg/L) and sulphate (SO_4^{-2} = 579 mg/L), which can inhibit microalgae growth at high concentrations. Nevertheless, phenolic compounds and iron, which are greatly toxic for microalgae were found at low concentrations, 0.22 mg/L and 0.48 mg/L, respectively. In general, high levels of organic matter were not found: turbidity = 26.3 FTU, COD = 110 mg O_2/L, TOC = 22.1 mg/L and TN = 6.99 mg/L. Low concentrations of phosphorus in the form of inorganic salts (PO_4^{-3} = 0.40 mg/L) were also found.

Physicochemical characteristics of wastewaters resulting from the mixtures of UW and OMW are also recorded in Table 1 (5%OMW/95%UW and 10%OMW/90%UW, v/v). In view of the results it can be concluded that the addition of a higher proportion of OMW lead to an increase in most of the parameters studied, more significantly in the organic load.

The efficient growth of microalgae in wastewater is influenced by several factors such as temperature, pH, light availability and concentration of essential nutrients such as nitrogen, phosphorous and organic carbon among many others (Hodaifa et al., 2013). For this reason, wastewaters containing high organics, nitrogen and phosphorus sources have a higher potential towards microalgae cultivation and simultaneously microalgal wastewater treatment. For this reason, the supplementation of UW, with low organic load, with OMW, which contains a higher organic matter concentration could lead to an improvement of microalgal growth. In addition, the higher concentration of TN in the 100% UW medium could also enhance microalgal growth, since nitrogen is one of the major nutrients required for micralgae cultivation, as it constitutes about 1-10% of the microalgal biomass (Eze et al., 2018). The variation of OMW and UW may allow the development of a complete culture medium with all the nutrients required for microalgae growth (Hodaifa et al., 2013).

Table 1. Characterization of the wastewaters before and after *S. obliquus* cultures.

Parameters	Single wastewaters				Wastewaters mixtures used as culture			
	Raw OMW*	Raw UW	100% UW		5% OMW/95% UW (v/v)		10% OMW/90% UW (v/v)	
			Before	After	Before	After	Before	After
pH	6.12	-	8.2	8.6	8.25	6.65	7.93	6.92
Conductivity, mS/cm	1.99	1.32	1.47	1.75	1.39	4.46	1.41	3.48
Turbidity, FTU	32.9	26.3	2.18	49.7	0.90	14.4	0.57	5.1
COD, mg O_2/L	3746	110	74.5	85.1	227	692	231	319
TPCs, mg/L	21	0.22	0.05	0.04	1.19	0.18	3.38	0.31
TOC, mg/L	372	22.1	3.37	12.7	31.3	34.5	59.1	56.2
TC, mg/L	426	48.1	62.5	23.6	66.7	38.7	94.8	77.4
IC, mg/L	54.1	25.9	59.2	10.9	35.4	4.21	35.7	23.1
TN, mg/L	6.56	6.99	20.8	3.03	7.61	1.74	7.45	1.94
NN, mg/L	-	0.57	6.98	0.01	0.73	0	0.91	0
NH_4^+, mg/L	0.71	1.90	0.072	0.34	1.76	0.36	1.57	0.34
Cl^-, mg/L	580	202	246	245	286	231	292	300
SO_4^{-2}mg/L	320	579	667	421	702	436	667	869
PO_4^{-3}, mg/L	26.8	0.40	0.21	18.8	1.35	0.7	2.35	0.33
K^+, mg/L	24.4	2.30	2	27	18.4	-	-	-
Na^+, mg/L	-	-	1.73	1.04	-	-	-	-
Fe, mg/L	0.71	0.48	0.011	0.53	0.28	0.13	0.40	0.31

**OMW treated by flocculation and photolysis with artificial UV light.*

4.3.3.2. *Scenedesmus obliquus* growth

Fig. 1 shows a sample of the growth curves of *S. obliquus* when the microalgae was grown in the culture medium formed by 5% OMW and 95% UW (v/v). In none of the experiments a lag phase was observed at the beginning of the cultures. Adaptation phase is a period in which microalgae get adapted to a new environment, this phase must be as short as possible to improve biomass productivity (Liao et al., 2018).

In all the experiments, the exponential was the first growth phase observed with a duration which ranged from 167 h (10%OMW/90%UW, v/v) to 235 h (5%OMW/95%UW, v/v). This phase is characterized by the availability of all nutrients required for microalgal biomass accumulation, with carbon, nitrogen and light as the most essential compounds (Liao et al., 2018).

The determination of the maximum specific growth rate of *S. obliquus* was determined during this phase according to equation (1),

$$\ln (x/x_o) = \mu_m t + a \quad (1)$$

where 'μ_m' is the slope of the line and corresponds to the maximum specific growth rate and 'a' is the intercept.

The highest value of μ_m was achieved when 100% UW was used as culture media (μ_m = 0.0202 h^{-1}), followed by the mixture with 5%OMW/95%UW, v/v (μ_m = 0.0138 h^{-1}) and by last, culture media with 10%OMW/90%UW, v/v (μ_m = 0.0122 h^{-1}).

A phase with linear increase of the biomass over time was observed after the exponential phase with a duration which ranged from 22.5 h (100% UW) to 431 h (5%OMW/95%UW, v/v). This phase is determined by the limitation of one or more nutrients such as CO_2 or light. In all experiments the CO_2 supply was performed through constant aeration with air at 0.5 L min^{-1} and light intensity was constant and equal to 359 μE m^{-2} s^{-1}.

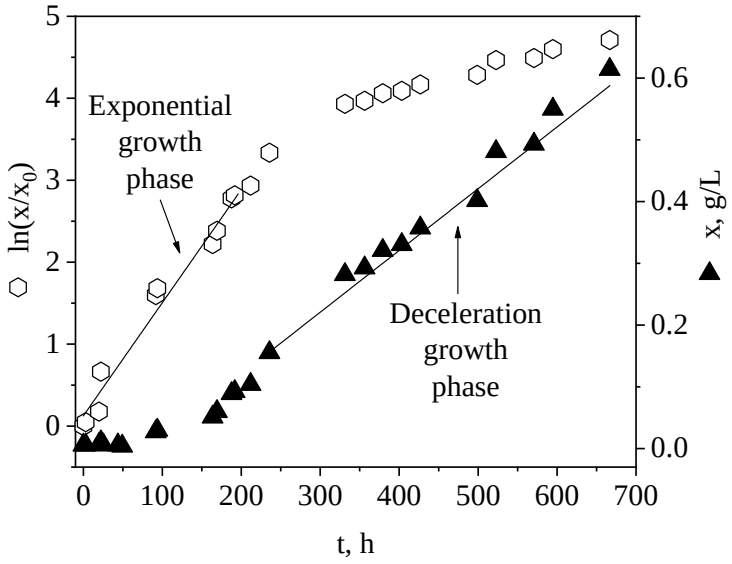

Fig. 1. Graphical determination of maximum specific growth rate and volumetric biomass productivity. Operating conditions: Culture medium = 5%OMW/95%UW (v/v), agitation rate = 200 rpm, T = 25 ºC, aeration rate = 0.5 L min^{-1} and illumination intensity = 359 μE m^{-2} s^{-1}.

The volumetric biomass productivity (P_b) was determined during the linear growth phase according to equation (2),

$$x = P_b\, t + a \qquad (2)$$

where 'P_b' is the slope of line and corresponds to the volumetric biomass productivity and 'b' is the intercept.

Similar values of biomass productivities were obtained in all experiments, ranging from 1.03 mg/(L h), (5%OMW/95%UW, v/v) to 1.21 mg/(L h), (100% UW) .

Finally, a stationary phase as well as the onset of cell death was observed at the end of the experiments. This phase is related to nutrients-starvation conditions. After reaching a peak point in microalgae biomass concentration, this phase is characterized by the accumulation of intracellular energy-storage compounds rather than biomass (Liao et al., 2018).

Fig. 2 shows the variation of all carbon (A) and nitrogen (B) species concentrations with time in the microalgal biomass from the 5%OMW/95%UW (v/v) culture medium. It can be observed in Fig. 2A a TOC increase in biomass along the culture explained by the ability of *S. obliquus* to take organic carbon from the culture medium and fix it and incorporate it into biomass structures, which resulted in an increment of the TC and TOC concentration in the biomass along the culture. IC levels in the biomass showed a little, almost negligible, rise along the culture.

It can also be observed in Fig. 2B a rapid increase in TN concentration during the starting period, particularly in the first 200 h, corresponding this increment with the exponential growth phase of the microalgae. This proved that nitrogen consumption was associated with microalgal growth and its conversion into biomass structures, mainly proteins. Once *S. obliquus* growth was stopped, the concentration of TN in the biomass remained constant until the end of the culture. No nitrate-nitrite was found in the biomass.

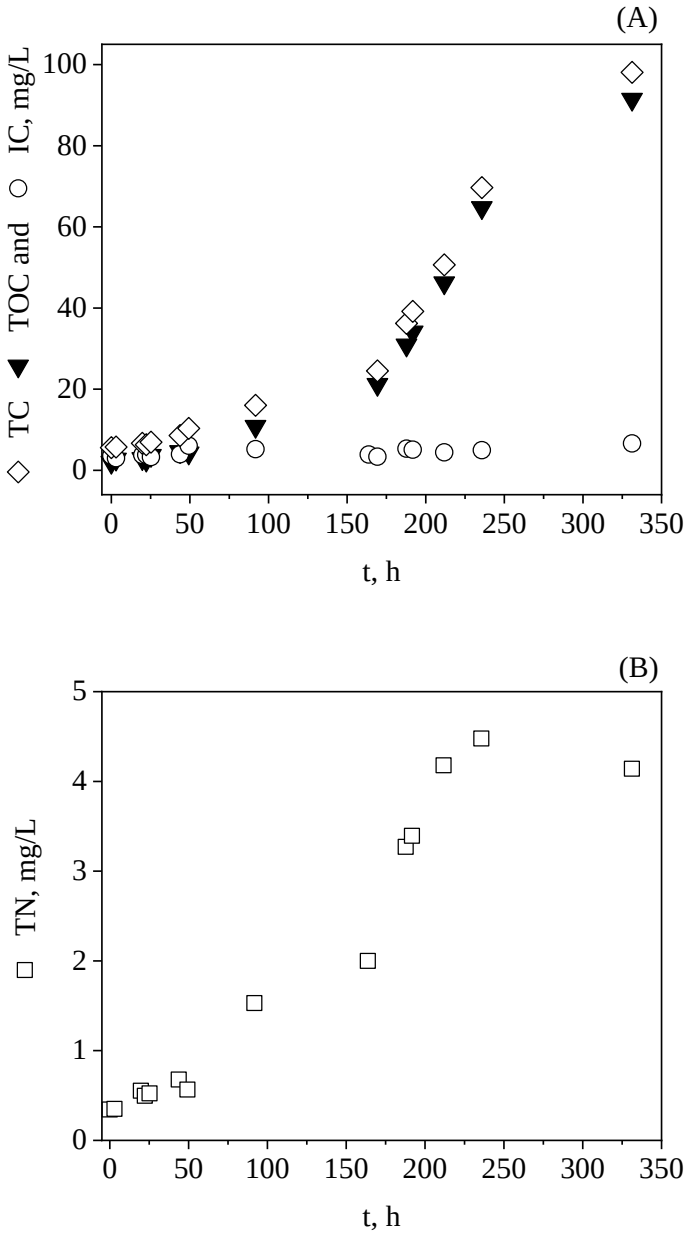

Fig. 2. Variation of total carbon species (A) and total nitrogen (B) on *Scenedesmus obliquus* biomass from the culture in the 5%OMW/95%UW (v/v) medium.

4.3.3.3. Biochemical composition of *S. obliquus* biomass

The biochemical composition of the biomass at the end of the experiments was influenced by the culture media composition. At the end of each experiment, the harvested biomass of *S.obliquus* was analyzed and the proteins, carbohydrates and lipids content was determined. In addition, total pigments (total chlorophylls and total carotenoids) were determined along the cultures. These are the microalgae cells main components. The variation of the biomass composition of *S. obliquus* for all culture media studied is shown in Table 2.

Comparing the protein content obtained in the biomass under the different culture conditions it was found that the highest value was achieved when 100% UW was used (initial $TN_{culture\ medium}$ = 20.8 mg/L and protein yield = 57.7%). The main compound required by microalgae for protein synthesis is the nitrogen, for this reason, a higher nitrogen concentration in the culture media can lead to further microalgae protein content. Protein yields of 4.06% and 7.54% were obtained in the 5%OMW/95%UW and 10%OMW/90%UW (v/v) culture media, respectively.

These results are consistent with the lipid yields obtained. Microalgae tend to accumulate lipids under stress conditions, such as nitrogen starvation. The initial TN concentrations in the 5%OMW/95%UW and 10%OMW/90%UW (v/v) media were 7.61 mg/L and 7.45 mg/L, respectively. In contrast, the initial TN concentration was equal to 20.8 mg/L in 100% UW. In this sense, the lowest % lipid was obtained in the biomass from the 100% UW medium, equal to 3.16%, in comparison with the highest lipid content, equal to 19.7%, obtained in the biomass from the 5%OMW/95%UW (v/v) medium. The obtaining of a high lipid fraction in the final biomass gives rise to the possibility of using this fraction for biodiesel production.

Carbohydrate content increased at lower nitrogen concentrations in the culture media, which is consistent with previous findings showing that carbohydrate accumulation in microalgae is triggered by nitrogen depletion (Wang et al., 2015). 37.2%, 75.2% and 75.3% of carbohydrates were obtained in the biomass from the 100%UW, 5%OMW/95%UW (v/v) and 10%OMW/90%UW (v/v) culture media, respectively. These high values are also indicative of the nitrogen deficiency, which resulted in the accumulation of organic compounds such as

polysaccharides by the microalgae. Biomass with high carbohydrates content is suitable for its use in biofuels generation (Gouveia and Oliveira, 2009).

In view of the biochemical composition results it can be concluded that *S. obliquus* is a versatile microalga capable of adapting its biochemical composition to the culture media and the availability of nutrients.

Table 2. Metabolites yields (% dry cell weight) of *Scenedesmus obliquus* final biomass.

Culture medium	Proteins %	Lipids %	Pigments %	Carbohydrates %
100%UW	57.7	3.16	0.94	37.2
5%OMW/95%UW (v/v)	4.06	19.7	0.06	75.2
10%OMW/90%UW (v/v)	7.54	15.9	0.22	75.3

4.3.3.4. Wastewater degradation by microalgae and final treated water quality

Microalgae can consume inorganic and organic nutrients from wastewaters for cell generation. Fig. 3A shows the variation of all carbon species concentration with time in the treated OMW without microalgae (culture medium). It can be observed a TOC slight decrease during the first 200 h of the culture, followed by an increment of TOC and TC in the last stages of the culture, explained by cell death and ruptures, which lead to an increase in the content of organic compounds in the medium. In all experiments, IC concentration was also decreased with time. As it happened with TOC, the highest reduction levels of the IC concentration occurred during the first 200 h of the culture, which can be explained by the ability of *S. obliquus* to grow mixotrophically assimilating organic compounds and CO_2 as carbon sources while using inorganic compounds as electron donors when there was light availability (Chojnacka and Marquez-Rocha, 2004).

Fig. 3B shows the variation of total nitrogen species in the culture media along the culture. It can be observed a decline in the total nitrogen concentration during the first stages, corresponding the most pronounced decrease with the exponential growth of *S. obliquus*. This proved that nitrogen consumption was associated with microalgal growth and its conversion into

biomass structures, mainly proteins. Once the exponential and lineal growth were finished, the concentration of TN in the culture medium remained constant, which can be explained by the cessation of nitrogen assimilation when TN concentration in the culture media was below 2.5 mg/L, corresponding this cessation with the beginning of the stationary phase of growth. Proteins are essential for microalgae growth. Nutrient deficiency, such as nitrogen starvation, could inhibit protein synthesis and microalgae growth subsequently.

In the experiments, the difference between the total nitrogen concentrations at the beginning and at the end of the culture corresponded to the nitrogen assimilated by *S. obliquus*. This nitrogen removal ranged from 77.1% (5%OMW/95%UW, v/v) to 85.4% (100% UW, v/v). It can also be seen a reduction in the nitrate-nitrite concentration along the culture, which was completely consumed after 169 hours, which means that all NN present in the culture medium was assimilated by *S.obliquus*.

These results proved the ability of *S. obliquus* to remove and assimilate pollutants as nitrogen in different forms such as nitrate, nitrite, or ammonium. This has the mutual advantage of diminishing the harmful effects of wastewaters as well as the reduction of the eutrophication effect in aquatic environments, caused mainly by nitrogen, phosphorus and carbon (Delgadillo-Mirquez et al., 2016). This has been proved by several authors such as Wang et al. (2015) who reported ammonium removal levels of up to 83% for several microalgae species.

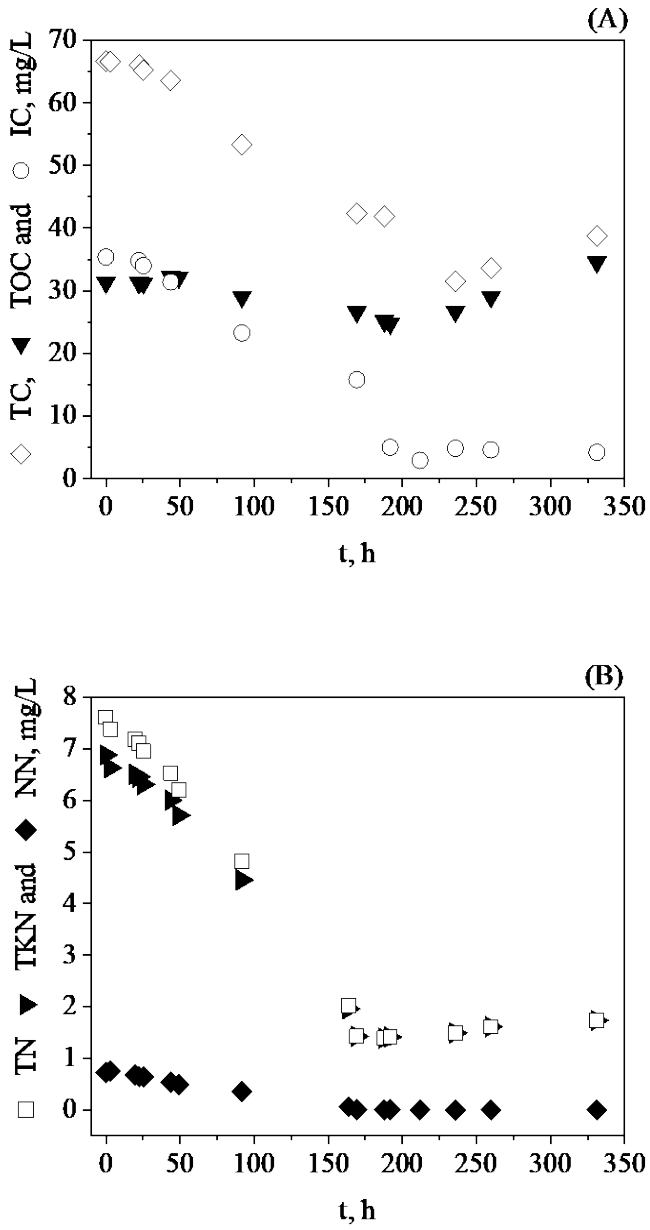

Fig. 3. Variation of total carbon species (A) and total nitrogen (B) on the treated culture medium (without algal biomass) formed by 5%OMW/95%UW (v/v).

Table 1 shows the treated water characteristics after microalgae growth. In general, most of the studied parameters were decreased throughout *S.obliquus* culture in both wastewaters mixtures, with some exceptions such as turbidity, COD or TOC due to the presence of cell debris in the final treated water as well as cell ruptures, which caused an increase of these parameters after *S. obliquus* culture. The highest removal percentages in the 5%OMW/95%UW (v/v) culture medium were obtained for NN (100%), IC (88.1%), TPCs (84.9%) and TN (77.1%). In the case of the 10%OMW/90%UW (v/v) medium, the highest values were achieved for NN (100%), TPCs (90.8%), NH_4^+ (78.4%) and PO_4^{-3} (85.9%).

With respect to 100% UW characterization, it can be observed that *S. obliquus* culture allowed high removal percentages of most parameters. The highest elimination values were obtained for TPCs (20%), IC (81.6%), TN (85.4%) and NN (99.9%). On the other hand, the increase in the concentration of some parameters after secondary treatment (*S.obliquus* culture) can be explained by the presence of organic matter in the culture media at the end of the culture as a consequence of cell ruptures during the last stages of *S. obliquus* growth.

4.3.4. Conclusions

Scenedesmus obliquus can assimilate nutrients from wastewaters. This enables the use of wastewaters as microalgal culture media with the mutual advantage of wastewater treatment and high added value biomass generation. Urban wastewater and olive oil mill wastewater have a complex composition which hampers its treatment as well as the microalgal growth, since microalgae require a proper nutrient composition in the culture media with carbon, nitrogen and phosphorous sources as the most essential components for biomass generation. In this sense, the mixture of OMW and UW allowed the development of a complete culture medium with all the nutrients required for microalgae growth. Nevertheless, the low protein yields and high carbohydrates content of the final biomass confirmed a nutritional stress situation associated with nitrogen limitation.

The final biomass obtained in the OMW and UW mixtures was characterized by high values of carbohydrate and lipid contents, which could lead to the production of biofuels.

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4.4. DETERMINATION OF THE THERMAL OXIDATION STABILITY AND THE KINETIC PARAMETERS OF COMMERCIAL EXTRA VIRGIN OLIVE OILS FROM DIFFERENT VARIETIES

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ABSTRACT

The use of olive oil with cooking purposes, as final seasoning or within cooked foods is increasing worldwide due to its numerous nutritional and health benefits. These attributes are mainly determined by olive oil chemical composition, which can be altered after thermal processing, oxidation processes or incorrect practices. For this reason, and due to the numerous factors which have influence in olive oil quality, it is highly relevant its correct chemical characterization. In this study, fatty acid composition of four Extra Virgin Olive Oil (EVOO) varieties were studied. The major fatty acid (FA) determined was oleic acid (77.1% on average), followed by palmitic (11.5% on average). In addition, thermal oxidation behaviour of the four EVOO samples was studied as an indicator of their quality and stability during thermal processing. This was performed through Differential Scanning Calorimetry (DSC) from a temperature of 40 °C at six different heating rates in the range of 0.5–10 °C min^{-1}. DSC records showed the same pattern and a small shoulder in the thermooxidation peak was present for all samples and all heating rates. The presence of initial and final oxidation products (by monitoring K_{232} and K_{270} values, respectively) was discarded according to the International Olive Council method.

Keywords: Differential Scanning Calorimetry (DSC); Extra Virgin Olive Oil (EVOO); Oxidation Onset Temperature (OOT); Oxidation Induction Time (OIT); Specific UV extinction coefficients.

4.4.1. Introduction

Nowadays, 85% of the total fats consumed in the Mediterranean diet comes from olive oil, a vegetable oil whose consumption is associated with several health benefits such as lower incidence of cardiovascular diseases, cancer and increased longevity (Perona and Botham, 2013). Most attributes of olive oil quality are determined by its chemical composition as well as the biochemical status of the olive fruit. To produce high-quality oil, the olives must be harvested without breaking the skins, and they must be processed within 12-24 hours of harvest (Calabriso et al., 2015). Extraction must be made from healthy fruits, avoiding manipulation or treatments which could alter the chemical composition of olive oil during the extraction and storage process (Oliveras López, 2005). In addition to olive picking, storage and processing, olive oil composition is determined by olive tree cultivation, climate, geographical area, etc. (Calabriso et al., 2015). This make every batch unique and difficult to standardize experimental conditions (Santos et al., 2013).

The group of major compounds in olive oil composition are triglycerides, which constitute between 92–98%. It also contains fatty acids, which contribute 94–96% of the total weight of triglycerides. In this fraction, six are major compounds: oleic (55.2-86.6%), palmitic (6.30-20.9%), linoleic (2.7-20.2%), stearic (0.32-5.33%), palmitoleic (0.32-3.52%), and linolenic (0.11-1.52%). Olive oil is also composed by minor components, fraction constituted by compounds, which derive from triglycerides, and liposoluble compounds. This minority fraction can be grouped in: diacylglycerols (DAGs), monoacylglycerols (MAGs), free fatty acids (FFAs), oxygenated fatty acids (OFAs), cyclic fatty acids, nonlinear FAs (branched FAs), dimeric FAs and another compounds, such phenols and pigments. The total of these compounds represents between 2-5% of the total composition (Perona and Botham, 2013)

Olive oil is commonly used as final seasoning, but it is also used with cooking purposes at high temperatures. In this sense, after thermal processing, changes and degradation processes are expected in olive oil, the most usual changes consist of triglyceride polymerization and hydrolysis, fatty acid and sterol oxidation and Maillard reactions (Santos et al., 2013). Oxidation can also alter the flavour and nutritional quality of olive oil due to the loss of beneficial substances and the generation of new toxic compounds including oxidized fatty acids, sterols or TAG polymers, which

can have a possible impact on human health and make olive oil less acceptable or unacceptable to consumers (Boskou, 2010). In this sense, Differential Scanning Calorimetry (DSC) is a technique based on the measurement of the energy changes that take place when a sample is heated, cooled or held isothermally, as well as the determination of the temperature at which these changes occur. These measurements enable the characterization of samples for several complex events such as melting processes or glass transitions (Gabbot, 2008). Although DSC has not been established by the International Olive Council as an official method for the determination quality, variety and geographical origin of olive oil. It has been suggested as a possible method with the advantages of being a fast and easy technique without the necessity of sample pre-treatment or use of solvents (Tan and Che Man, 1999; Ferrari et al., 2007). According to the official definition, extra virgin olive oil must be extracted by cold and mechanic conditions in an oxygen free atmosphere to preserve the naturally present antioxidants. In refined olive oil, antioxidants are degraded due to refining processes and high temperatures during the olive oil production; as a consequence, the induction period is shorter in lower quality olive oils and can be used to study and compare the thermooxidative stability of samples (Cibulková et al., 2014). In this sense, the oxidation of edible oils exhibits the induction period and at the end of the induction period, the quality of the oil suddenly deteriorates so that the induction period is considered as a measurement of the oil stability (Šimon and Cvengroš, 2010).

In addition to DSC, spectroscopic techniques are suitable for quality control of olive oil. Fluorescence spectroscopy is a simple, rapid, economic and non-destructive technique which is applied to determine the stage of decomposition of oils (Guzmán et al., 2015). The K_{232} and K_{270} values are spectrophotometric measures for quantifying the UV absorption at 232 nm and 270 nm, respectively. It provides information about the quality of the fat, the conservation status of the oil and any deterioration occurred during the technological processes (Calabriso et al., 2015). It corresponds to the maximum absorption of the conjugated dienes and trienes and it is expressed as specific extinctions coefficients (Alouache et al., 2015).

Other technique that can be found in the literature is 'Rancimat stability' which consist of exposing the olive oil to forced oxidation at 100 ºC until its maximum oxidation, measuring the

time required for an abrupt change in conductivity from an aqueous solution where the volatile compounds carried by the oil were collected. The duration time of this period is considered as the index of resistance to rancidness of the fat being assayed (Nieto et al., 2010).

In this work, the quality and stability of different varieties of olive oil were studied. The fatty acid profiles of four commercial EVOO were determined. The thermal oxidation stability and the kinetic parameters related to the oxidation process by DSC were evaluated. The specific UV extinction coefficients (K_{232} and K_{270}) were determined to study the presence of oxidation products.

4.4.2. Materials and Methods

4.4.2.1. Samples

Four extra virgin olive oils samples of different brands were bought in a local store in Spain (Table 1). The samples were kept in a refrigerator at 4°C until the time of analysis.

Table 1. Identification of extra virgin olive oil samples analysed.

Variety	ID	Origin
Coupage Changlot Real and Arbosana	C+A	Spain
Manzanilla cacereña	Ma	Spain
Koroneiki	Ko	Greece*
Arbequina	Ar	Spain

**Olives grown in Spain.*

4.4.2.2. Fatty acid profiles determination

A mass between 0.10 and 0.30 g of each sample was weighted and dissolved in heptane in a reaction vessel with volume capacity equal to 1 cm^3. After the sample dilution, 100 µl of sodium methoxide, the transesterification agent, was added. The time of the transesterification reaction had a duration between 15 and 20 minutes. Then, an excess of methanolic HCl (typically 100 µl) was

added and the reaction was carried out at room temperature for 45 minutes. The upper heptane layer was separated and injected into the gas chromatograph (Christopherson and Glass, 1969).

Fatty acid composition was determined by the gas chromatograph GC-7890 (Agilent, USA) with a FID detector and capillary column DB-23 (60 m x mm x 00:25 12:25 film microns). A volume of 1 mL of FAME and heptane was injected. Carrier gas flow rate was equal to 16.4 cm^3 min^{-1} and pressure = 220 kPa. Programming chromatographic temperature was set at the initial value of 150 °C (held for 6 min), followed by a heating rate of 5 °C min^{-1} up to 170 °C and heating rate of 6 °C min^{-1} up to 220 °C (held for 6 min). Next stage was a heating rate of 6 °C min^{-1} at 220 °C for 1 min and finally, heating rate of 30 °C min^{-1} up to 240 °C for 10 minutes. FID hydrogen flow and airflow rate were 40 cm^3 min^{-1} and 450 cm^3 min^{-1}, respectively.

4.4.2.3. Differential Scanning Calorimetry

The DSC analysis was conducted on a differential scanning calorimeter, Shimadzu DSC-60 (Tokyo, Japan) equipped with an automatic gas switching unit. The temperature scale of the instrument was calibrated to the melting points of benzil, In, Sn, and Pb. The measurement of thermooxidative stability was carried out in non-isothermal mode with linear heating. Samples of 3.5–4.5 mg were placed into open aluminium pans and heated in dynamic air atmosphere (50 mL min^{-1}) from 40 °C at 6 different heating rates in the range of 0.5–10 °C min^{-1}. Each measurement was terminated once an exothermic peak corresponding to thermal oxidation was observed.

4.4.2.4. Determination of specific UV extinction coefficients (K_{232} and K_{270})

The measurement was performed through UV/VIS spectrophotometry with a UV-1600 series spectrophotometer (VWR, Leuven, Belgium). Absorbance within a 200 to 800 nm spectral range was measured at 1 nm spectral resolution using a 1 cm path length quartz cell, in the region of 200-380 nm.

Olive oil samples were perfectly homogeneous without any suspended impurities. A mass of 0.25-0.30 g was weighted and diluted to a one percent solution in cyclohexane. Spectrophotometric analysis of olive oil in accordance with the official method in the Commission

Regulation (EC, 2000), which involves the determination of the specific extinction in cyclohexane at wavelength of 232 and 270 nm, and the determination of K_{232} and K_{270} according to eq. (1).

$$K_\lambda = A_\lambda/(c \cdot L) \quad (1)$$

where, K_λ is the extinction coefficient, A_λ is the absorbance, c is the concentration of the sample in the solvent in g/100 mL, and L is the path length of the cuvette in cm.

4.4.3. Results and discussion

4.4.3.1. Fatty acids composition of extra virgin olive oils

The fatty acid (FA) profile of olive oil is highly relevant and it is considered as a parameter to characterize the diverse olive varieties since the quality of the fat has a direct impact on oil quality and thus, on consumer health (Rueda et al., 2014). In addition to the clinical relevance and the nutritional value of some FA such as oleic acid, FA are also responsible for the presence of desired and undesired volatile compounds, which have a direct influence on the positive or negative sensory perceptions in olive oil. Lipoxygenase (LOX) pathways generate most of the desired volatile aroma compounds (C5 and C6 compounds, saturated aldehydes etc.) A series of oxidative reactions that result in a large variety of metabolites from polyunsaturated FA, being linoleic and linolenic acids the main initial substrates. The importance of the FA profile is, therefore, because high- and poor-quality olive oils differ by their content in these compounds derived from FA (Reboredo-Rodríguez et al., 2016).

Fatty acid content of olive oils is highly variable since it is affected by numerous factors such as production and cultivation area, latitude, climate, fruit ripeness, genetic factors etc. Environmental factors are the one that have a greater influence on FA composition of olive oils, being temperature the one that plays an essential role in the FA profile of olive oil, since temperature regulates fatty acid desaturases. Polyunsaturated fatty acids are present in greater proportions at low temperatures (Hernández et al., 2011). In this sense, differences in the FA profile of the four studied EVOO can be explained by the different geographical areas and climate conditions in which olive fruits were grown. In addition, several agronomic, processing and environmental variables such as

degree of ripeness or storage and processing conditions have a direct influence on the olive oil chemical composition (Aparicio and Luna, 2002).

Table 2 shows the fatty acid profile (%, weight) of the different EVOO. Determined fatty acids have been grouped as total saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. The major fatty acid percentage found was oleic acid (C18:1) as expected. This fatty acid content ranged from 75.2% (Ar) to 79.9% (Ko). Followed by palmitic acid (C16:0) which ranged from 10.4% (Ko) to 12.9% (Ar), linoleic acid (C18:2), from 5.09% (Ko) to 8.27% (Ar), stearic acid (C18:0), which ranged from 1.85% in Ar to 2.08% in C+A and linolenic acid (C18:3) whose content ranged from 0.59% in Ar to 2.82% in C+A. Other fatty acids such as palmitoleic acid (C16:1, 0.86% on average), gadoleic acid (C20:1; 1.24% on average), behenic acid (C22:0; 0.50% on average) and arachidic acid (C20:0; 0.27% on average) were detected in all EVOO samples and found at a concentration of less than 1%. In general, no significant variation was detected in the fatty acids composition of the different EVOO studied, showed by the standard deviation values, which varied from 0.10 (C20:0) to 2.23 (C18:1). Saturated fatty acids comprised about 13.6% of the total fatty acids, whereas monounsaturated and polyunsaturated fatty acids represented 77.4% and 8.98%, respectively. Total unsaturated fatty acids (MUFA + PUFA) in olive oil constituted 86.4% of the total. These fractions corresponded, almost entirely, to oleic acid, while palmitic acid represented the greatest proportion of SFA.

Regarding FA composition, significant differences exist between olive oil and other vegetable oils. In this sense, Li et al. (2018) determined the fatty acid profile of palm oil, rapeseed oil, sunflower oil, and linseed oil. Compared to these four vegetables oils, it must be highlighted the higher oleic acid content in the four EVOO studied in this work (77.1% in average) in comparison with rapeseed, palm, sunflower and linseed oil, whose content in oleic acid were notably lower: 46.3%, 33.6%, 13.6% and 1.2%, respectively. In addition, palmitic acid, the second most abundant FA in olive oil (11.5% on average), was found in notably lower percentages in sunflower oil (3.89%), linseed oil (3.12%) and rapeseed oil (2.69%), nevertheless, higher content of this FA was found in palm oil (29.3%) in comparison with EVOO. Content of linoleic and stearic acids in EVOO (6.44% and 1.99% on average, respectively) were lower in comparison with the other vegetable oils, whose

content ranged from 8.12% (palm oil) to 51.9% (sunflower oil) for linoleic acid and between 1.51% (rapeseed oil) and 3.59% (palm oil) for stearic acid. Linolenic acid was only found in rapeseed and linseed oil, at a concentration of less than 1%. Myristic acid (C14:0), which was not found in olive oil, was found at a 0.43% in palm oil.

Table 2. Fatty acids profile determined in four commercial samples of EVOO.

Fatty acids	EVOO sample				Average	SD
	C+A	Ma	Ko	Ar		
C16:0 (palmitic)	11.2	11.6	10.4	12.9	11.5	1.03
C16:1 (palmitoleic)	0.80	0.88	0.67	1.08	0.86	0.17
C18:0 (stearic)	2.08	1.97	2.05	1.85	1.99	0.11
C18:1 (oleic)	75.4	77.7	79.9	75.2	77.1	2.23
C18:2 (linoleic)	6.16	6.26	5.09	8.27	6.44	1.33
C20:0 (arachidic)	0.33	0.36	0.28	0.13	0.27	0.10
C20:1 (gadoleic)	1.24	n.d	n.d	n.d	1.24	
C18:3 (linolenic)	2.82	0.84	0.89	0.59	1.29	1.03
C22:0 (behenic)	n.d	0.36	0.65	n.d	0.50	0.20
ΣSFA*	13.6	14.3	13.4	14.9	14.1	0.67
ΣMUFA**	77.4	78.6	80.6	76.3	78.2	1.84
ΣPUFA***	8.98	7.10	5.98	8.85	7.73	1.45

**Corresponding to the sum of saturated fatty acids.*
*** Corresponding to the sum of monounsaturated fatty acids.*
**** Corresponding to the sum of polyunsaturated fatty acids.*

Similarly, Berasategi et al. (2012) studied avocado oil fatty acid composition. This oil consumption and production is significantly growing in recent years due to its beneficial health properties attributed to its high concentration of oleic acid, antioxidant vitamins and phytosterols. This study showed that MUFA content in avocado oil was equal to 68.4% with a total content of 54.4% of oleic acid of total FA. These values are much lower in comparison with the EVOO studied in this work, which contained 78.2% on average of MUFA and oleic acid ranging from 75.2% to 79.9%. On the contrary, palmitoleic acid, whose average content in EVOO was equal to 0.86%,

was found at higher concentration (7.88%) in avocado oil. The importance of MUFA content can be explained by its relationship with higher concentration of minor compounds with antioxidant and hipocholesterolemic effects (Berasategi et al., 2012).

On the other hand, higher PUFA content was found in avocado oil (11.8%) in comparison with EVOO (7.73%). Within this group, EVOO contained 2-fold the amount of linolenic acid present in avocado oil (0.61%). Lastly, SFA content in avocado was equal to 11.8% in comparison with 7.73% in EVOO and with the main differences in palmitic and stearic acids, whose content were equal to 18.7 and 0.51% respectively.

4.4.3.2. Differential Scanning Calorimetry

The standard tests used for the determination of the induction period are predominantly carried out under isothermal conditions, i.e., the oxidation induction time is measured. However, under isothermal conditions, the oxidation peak measured is often flat and its onset, corresponding to the end of induction period, cannot be determined unambiguously. On the contrary, in the experiments with constant heating rate, the oxidation peak is distinct and the onset oxidation temperature can be measured accurately and unambiguously. In our previous work, a theory of the kinetic description of induction periods from non-isothermal measurements has been outlined (Šimon, 2005) and applied for the study of thermooxidation of edible oils (Šimon and Cvengroš, 2010). For the treatment of experimental DSC data, it was applied the procedure from the latter citation.

The DSC records of non-isothermal thermooxidation of olive oil C+A are depicted in Figure 1; the other EVOOs studied exhibited similar pattern. The peak corresponding to thermooxidation, which exhibits a small shoulder near its onset. The shoulder is present for all samples and for all heating rates employed; therefore, the values of oxidation onset temperatures, T_i, were evaluated as its onset extrapolated to the baseline. It can be seen from Figure 1 that higher heating rate always leads to higher oxidation onset temperature. Šimon (2005) demonstrated that employing a non-Arrhenian dependence of the reaction rate on temperature, $k(T) = A' \exp(DT)$, and

assuming the same conversion for all heating rates, the dependence of oxidation onset temperature (T_i) on the heating rate can be described by equation (2),

$$T_i = \frac{1}{D}\ln(A\ D\ \beta + 1) \tag{2}$$

where, 'β' is the heating rate in °C min^{-1} and 'A' and 'D' are kinetic parameters of thermooxidation.

Once the values of the kinetic parameters are determined from a series of experiments carried out at different heating rates, the oxidation induction time (OIT) can be calculated as:

$$\mathrm{OIT(T)} = A \exp\ (-D\ T) \tag{3}$$

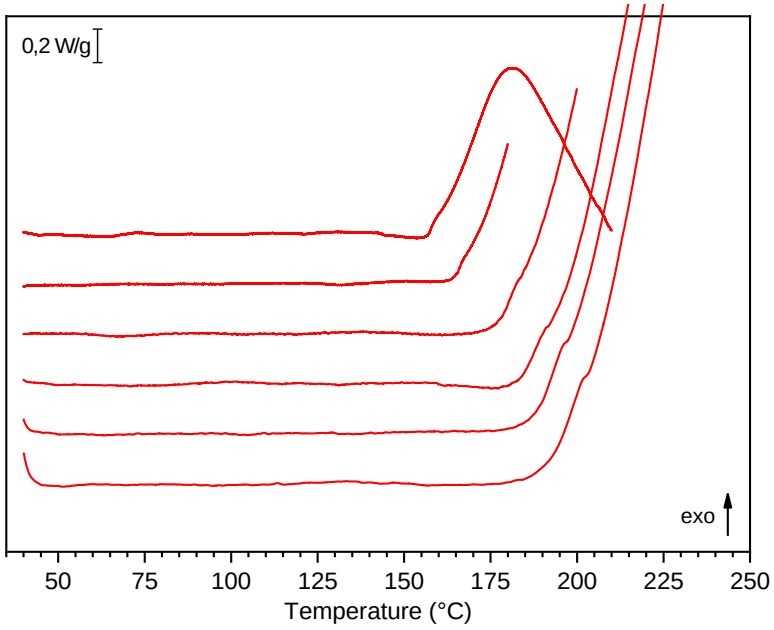

Fig. 1. Non-isothermal DSC records of thermal oxidation (olive oil: C+A) obtained for different heating rates (from *top to bottom*: 0.5, 1, 3, 5, 7, and 10 °C/min).

The evaluated oxidation onset temperatures for each oil at various heating rates are listed in Table 3. These T_i *vs.* β dependences were further analysed to estimate the kinetic parameters

employing non-linear least squares method applied to eq. (3); the resulting parameters are listed in Table 4. Figure 2 depicts a typical result of the least squares fitting procedure.

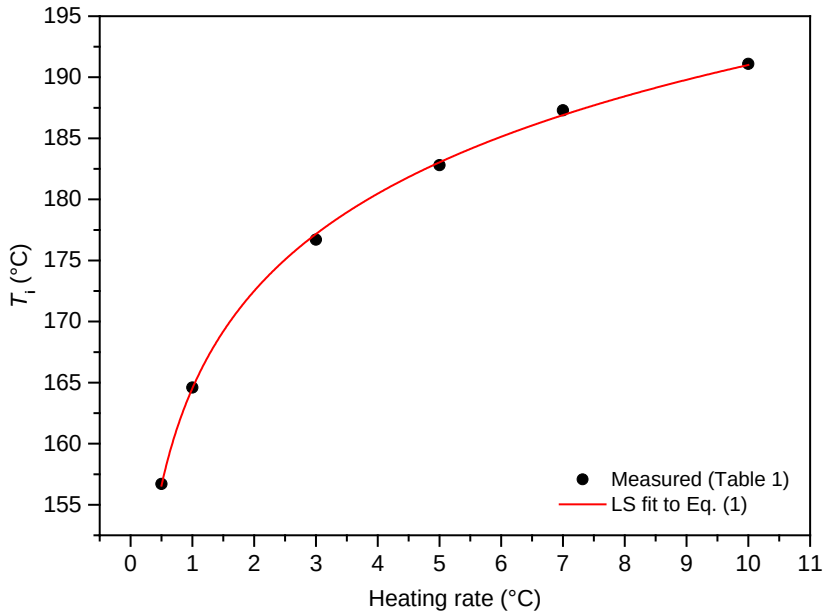

Fig. 2. Experimental and fitted dependences of the oxidation onset temperatures on the heating rate (olive oil: C+A).

Table 3. Oxidation onset temperatures of olive oils for various heating rates.

β (°C min^{-1})	T_i (°C)			
	C+A	Ma	Ko	Ar
0.5	156.7	156.2	158.0	152.3
1	164.6	165.3	167.8	161.3
3	176.7	176.0	180.5	174.9
5	182.8	181.6	189.0	182.6
7	187.3	187.9	193.3	186.0
10	191.1	192.9	196.9	190.0

The kinetic parameters obtained from the treatment of non-isothermal data were used to predict the values of OIT. The prediction of the values of oxidation induction time, OITs, based on eq. (3) for each olive oil are presented in Figure 3. Two representative temperatures were chosen

(25 °C and 150 °C). The lower temperature represents the usual storage conditions. However, care should be taken since both representative temperatures chosen (25 ºC and 150 ºC) are outside the experimental range of DSC measurements. The higher representative temperature chosen (150 ºC) is much closer to the experimentally investigated temperature range and the corresponding OIT values are expected to be both more precise and accurate.

Table 4. Values of the kinetic parameters with their standard errors.

	C+A	**Ma**	**Ko**	**Ar**
ln A/min	40.51 ± 0.43	39.47 ± 1.09	36.23 ± 0.80	36.70 ± 0.56
D (K^{-1})	0.08697 ± 0.00099	0.0846 ± 0.0024	0.0764 ± 0.0018	0.0786 ± 0.0013

Figure 3 shows that all the OITs values predicted at 150 °C lie in a relatively narrow range of 30 to 50 min with oil Arbequina being least stable. Considering the OITs uncertainty, all the olive oils exhibit approximately the same high temperature termooxidative stability.

Results for 25 °C also suggest that Arbequina is the least stable oil and the Coupage Changlot Real and Arbosana has about four times longer shelf life—the differences between the oils are now much more pronounced. However, it should be kept in mind that the temperature (25 °C) lies far away from the experimental range and non-linear extrapolation affects both accuracy and precision of the results (as demonstrated by much longer error bars compared to high-temperature prediction).

Similarly, Li et al. (2018) studied thermal oxidation stability of four different vegetable oils (palm, rapeseed, sunflower and linseed oil) through DCS at different heating rates (1, 5, 7.5, 10, 15, 20 °C/min). According to the T_i obtained for the different oils, the following order for oxidation stability was obtained: palm oil > rapeseed oil > sunflower oil > linseed oil. When comparing (Li et al. (2018) results with the present study, it can be concluded that for all heating rates, the four vegetable oils showed higher T_i in comparison with the EVOO studied in the present work. T_i at a heating rate of 10ºC/min was equal to 250.2, 233.3, 221.1 and 202.9 ºC for palm, rapeseed, sunflower and linseed oil, respectively. In contrast, T_i values between 190 and 196.9 ºC were

obtained for the EVOO samples at the same conditions. Similar pattern was observed for all heating rates. In addition, similar behaviour was registered in both studies when comparing thermal decomposition profiles at different heating rates: higher heating rate resulted in higher degradation rate and increased T_i.

Differences in oxidation stability of these vegetable oils are directly related to FA composition: vegetable oils with higher UFA content are usually less stable than those with higher SFA proportion. This can be explained by FA chemical structure, determined by chain length, unsaturation degree and ramifications. Oxidation mostly occurs in double bonds, for this reason, FA with higher unsaturation degree are more prone to oxidation and less stable, consequently, than SFA (Micić et al., 2015; Refaat, 2009).

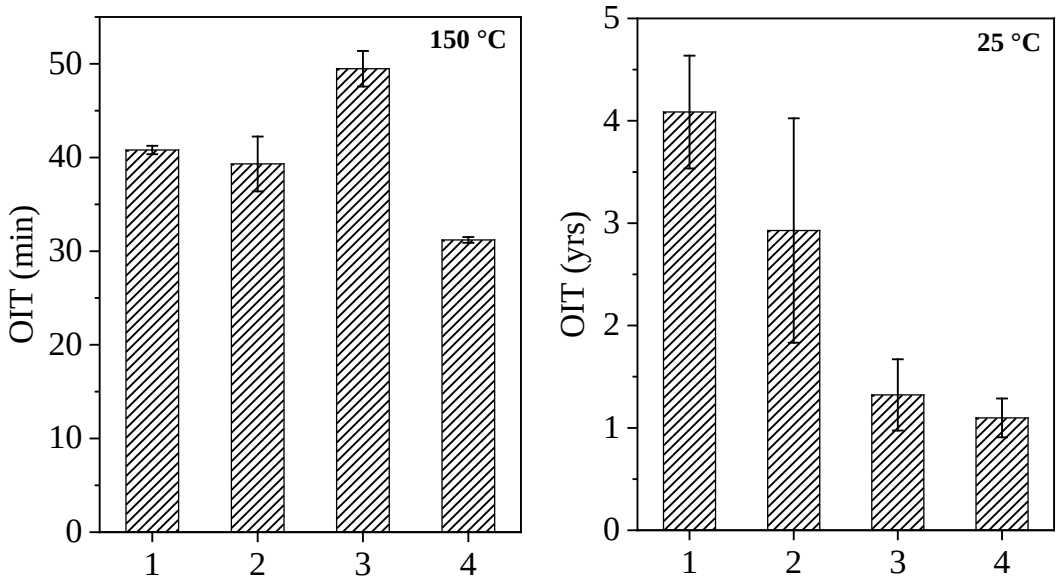

Fig. 3. OITs for olive oils 1) C+A, 2) Ma, 3) Ko and 4) Ar predicted from non-isothermal experiments using eq. (3).

4.4.3.3. Ultraviolet Spectrophotometry

The four EVOOs varieties studied showed similar UV spectra in the UV and visible range (Figure 4). Evaluation of the spectra of the four samples according to eq. (1) yield the values summarized in Table 5. As shown, all olive oils fulfil the criteria for extra virgin olive oil laid down

by the International Olive Oil Council and the Commission Regulation (Ec, 2000) since K_{232} and K_{270} values were lower than the limits established 2.50 and 0.22, respectively.

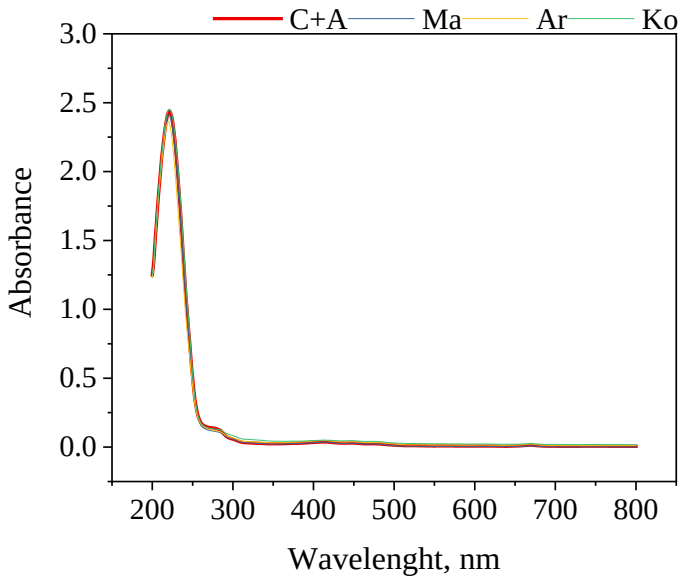

Fig. 4. UV spectra for the four olive oil varieties studied.

Table 5. K_{232} and K_{270} values of the analyzed EVOO samples.

	K_{232}	**K_{270}**
Extra virgin olive oil criteria*	**≤ 2.50**	**≤ 0.20**
Changlot Real + Arbosana	1.95	0.14
Manzanilla Cacereña	1.88	0.12
Koroneiki	1.71	0.13
Arbequina	2.02	0.14

**Maximum values allowed according the Commission Regulation (CEE) no. 2568/91: K_{232} ≤2.50 and K_{270} ≤0.20.*

K_{232} is related to the presence of hydroperoxides, conjugated dienes, carboxylic compounds and conjugated trienes. On the other hand, K_{270} is dependent on the secondary products formed from the oxidation products detected at 232 nm (Bouarroudj et al., 2016; Guzmán et al., 2015).

Therefore, results indicated the absence of oxidation products in the olive oils studied as well as the absence of refining oil in the commercial EVOO samples.

Allouche et al. (2007) studied the evolution of K_{232} and K_{270} values of two extra virgin olive oils from Arbequina and Picual cultivars during heating at 180 ºC. Results showed that both indexes increased notably during the heating process, obtaining the higher values for Arbequina oil. Similarly, it was experimentally proved by Guzmán et al. (2015) that during oil oxidation, high levels of peroxides are generated from primary oxidation compounds, resulting in higher K_{232} and K_{270} values and fluorescence spectra with peaks in the 415-600 nm region. In addition, it was demonstrated in this study that the combination of fluorescence techniques with multivariate analysis is a suitable method to characterize olive oil on the basis of the main quality parameters of olive oil: peroxide value, K_{232}, K_{270} and acidity.

The suitability of K_{232} and K_{270} to determine the quality and conservation status of vegetable oils was also proved by Rodrigues et al. (2015). In this work, oil from *Jatropha curcas L* seeds was stored for 42 days, at 35 ºC and 75% or 92% relative humidity (RH). Results showed that higher RH resulted in a higher increment in K_{232} and K_{270} values. Regarding K_{232}, an increase of 0.029 absorbance units/day was observed at 75% RH; nevertheless, a faster increase was observed at 92% RH (0.059 absorbance units/day). Similar results were obtained for K_{270}, showing an increase from 0.07 to 0.22 after storage in higher humidity conditions.

4.4.4. Conclusions

Authentication and traceability of extra virgin olive oils are highly in demand in the market. The International Olive Oil Council and the Commission Regulation (Ec, 2000) has defined the quality of olive oil according to a series of parameters such as free fatty acids content and UV specific extinction coefficients (K_{232} and K_{270}). These parameters were determined in this work; results showed that oleic acid is the most abundant in the four EVOO (77.1% on average), followed by palmitic (11.5% on average). The importance of FA profile is due to its high contribution to olive oil oxidative stability. K_{232} and K_{270} values confirmed the absence of oxidation primary and secondary products.

In addition, the results showed that oil analysis can be performed with Differential Scanning Calorimetry, an alternative technique for the evaluation of olive oil quality and stability as well as the determination of the heating effect on olive oil. DSC is an efficient, fast, accurate and environmentally friendly method for the identification of peaks related to olive oil chemical composition. Nevertheless, in terms of authenticity, the information provided by the DSC analysis is not enough to detect adulterated olive oils due to the large number of possible adulterants (Aparicio et al., 2013).

In the four different EVOO varieties studied, DSC provided thermal fingerprints of the samples. For all heating rates, the peak corresponding to thermooxidation exhibits a small shoulder near its onset and all samples shown similar DSC record. It also can be concluded from the analysis of the T_i vs. β dependences that, for all samples, higher heating rate always leads to higher oxidation onset temperature. When comparing results obtained at two representative temperatures (25°C and 150°C), higher temperature is much closer to the experimentally investigated temperature range, as a consequence, OIT values obtained are more precise and accurate, exhibiting all the oils approximately the same thermooxidative stability. Much longer error bars because of less accuracy and precision of the results are obtained at 25°C.

It can therefore be concluded that the control of storage conditions of olive oil (temperature, humidity, etc.) is extremely relevant to preserve its quality. Evaluation of FA profile, K_{232} and K_{270} values and T_i through DSC are suitable, simple and accurate techniques to predict the quality, conservation status and oxidation stability of different vegetable oils.

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5. CONCLUSIONES/CONCLUSIONS

A continuación, se procederá a mencionar las conclusiones finales obtenidas tras la realización de la parte experimental y la obtención de los resultados que han sido interpretados y discutidos. Por otra parte, y considerando la naturaleza de esta Tesis Doctoral que se presenta en forma de un compendio de artículos ya publicados, las conclusiones se dividirán en diferentes capítulos:

5.1. Proceso integral para el tratamiento de aguas residuales de almazara y su revalorización mediante la generación de biomasa microalgal de alto valor añadido.

5.1.1. Características fisicoquímicas de las aguas residuales de almazara brutas.

- Las aguas residuales de almazara (ARA) se caracterizaron por un elevado contenido en materia orgánica, determinado por los siguientes parámetros: turbidez = 714 FTU, demanda química de oxígeno (DQO = 5839 mg O_2/L), compuestos fenólicos totales (CFTs = 322 mg/L), carbono orgánico total (COT = 646 mg/L) y nitrógeno total (NT = 58,9 mg/L).
- Las ARA registraron una elevada concentración de compuestos fenólicos (CFTs = 322 mg/L). La estructura de estos, su fitotoxicidad y actividad antimicrobiana dificultan su degradación.
- Elevadas concentraciones de sales inorgánicas (carbón inorgánico = 318 mg/L) así como de fósforo en forma de sal inorgánica (ortofosfato = 43,1 mg/L) favorecen el crecimiento microalgal debido a su papel en el metabolismo de las microalgas.
- El elevado valor de la relación DQO/COT registrado (igual a 9) se debe a la elevada heterogeneidad de las ARA.

5.1.2. Efecto del tratamiento primario (floculación-sedimentación, fotólisis por luz UV artificial y microfiltración con membrana) en las características fisicoquímicas de las aguas residuales de almazara.

- El tratamiento primario global permitió la eliminación de una elevada carga orgánica, alcanzando porcentajes de eliminación del orden de 96,2%, 80,3% y 96,6% para la DQO, COT y CFTs, respectivamente.

- La operación de floculación-sedimentación con Flocudex CS-51 demostró ser la más efectiva en la eliminación de fenoles, con una eliminación del 78% respecto al 45,7% y el 72,7% alcanzados tras la fotólisis con luz ultravioleta durante 30 min y la microfiltración, respectivamente.
- En cuanto a la reducción en materia orgánica, la fotólisis con luz UV demostró ser la operación más efectiva. Los porcentajes de eliminación registrados tras la floculación, fotólisis con luz UV y microfiltración fueron 57,5%, 88,8% y 20,5% para la DQO y el 18%, 71,9% y 14,6% para el COT, respectivamente.

5.1.3. Crecimiento de *Chlorella pyrenoidosa* en agua residual de almazara pretratada y composición bioquímica de la biomasa final.

- Los valores más altos de la velocidad específica máxima de crecimiento (μ_m = 0,07 h^{-1}) y la productividad volumétrica en biomasa (P_b = 1,25 mg/(L h)) fueron obtenidos en el medio de cultivo con 50% de ARA (v/v).
- Para ambos parámetros cinéticos (μ_m y P_b) se registró un aumento en sus valores con el incremento de la concentración de ARA en el medio de cultivo, hasta una concentración del 50% ARA (v/v). A partir de este valor se observó una disminución de ambos valores debido al efecto inhibidor y tóxico de las ARA.
- La biomasa final cosechada presentó un elevado contenido en carbohidratos, con valores que variaron entre el 30,3% y el 89,2% en los cultivos con 100% y 5% ARA (v/v), respectivamente. Esto se debe a la capacidad de las microalgas de acumular compuestos energéticos en condiciones de estrés ambiental, tales como deficiencia de nitrógeno.
- El contenido de proteínas en la biomasa final se incrementó con el aumento de la concentración de ARA en el medio de cultivo hasta alcanzar un valor máximo del 51,5% en el medio con 100% ARA, debido a la mayor disponibilidad de nitrógeno.
- El mayor contenido en lípidos (34,2%) se registró en el cultivo con 25% de ARA (v/v). En cuanto a los ácidos grasos identificados en la fracción lipídica, fueron los saturados los más abundantes (85,2%-95,1%) y dentro de este grupo, el ácido palmítico (65,7%-74,7%).

5.1.4. Efecto del cultivo de *Chlorella pyrenoidosa* en las características fisicoquímicas de las aguas residuales de almazara.

- Los mayores porcentajes de eliminación fueron registrados en los medios de cultivo formados por una menor concentración de ARA, debido a la menor concentración de compuestos de inhibición en las mismas. Porcentajes de eliminación del 74,0%, 75,5%, 71,3% y 87,6% se obtuvieron para el CT, COT, CI y NT en el cultivo con 25% ARA (v/v).
- Los resultados experimentales revelaron que la velocidad de eliminación tanto de la materia orgánica (COT y NT) como del carbono inorgánico (CI) aumenta bruscamente durante la fase exponencial de crecimiento, lo que indica la capacidad de *C. pyrenoidosa* de crecer de forma mixotrófica asimilando compuestos orgánicos e inorgánicos como fuente de carbono.
- El mayor porcentaje de eliminación de compuestos fenólicos (67,1%) se registró en el cultivo con 50% ARA (v/v), disminuyendo a mayores concentraciones de ARA.
- Al final del proceso se obtuvo un agua tratada de alta calidad con unas características fisicoquímicas que la hacen apta para su reutilización en riego, actividades industriales o para su vertido en aguas receptoras.

5.2. Combinación de operaciones fisicoquímicas y cultivo de microalgas como un nuevo bioproceso para el tratamiento de las aguas residuales de almazara.

5.2.1. Caracterización fisicoquímica de las aguas residuales de almazara brutas.

- Las ARA registraron un elevado contenido en carga orgánica, principal parámetro a considerar desde el punto de vista medioambiental y determinado por los siguientes parámetros: turbidez = 714 FTU, DQO = 5839 mg O_2/L, CFTs = 322 mg/L, COT = 328 mg/L y NT = 58,9 mg/L.
- Las ARA cuentan con una baja concentración de nitrógeno total (NT = 58,9 mg/L) y de fósforo (PO_4^{-3} = 43,1 mg/L) respecto al medio sintético de control Rodríguez-López, con 140 mg/L y 160 mg/L, respectivamente.

5.2.2. Efecto del tratamiento primario (floculación-sedimentación y microfiltración con membrana) en las características fisicoquímicas de las aguas residuales de almazara.

- El tratamiento primario global demostró ser efectivo en la reducción de la mayoría de los parámetros estudiados, especialmente en la eliminación de sólidos totales, dando lugar a la reducción de compuestos de inhibición, turbidez y color.
- La etapa de floculación-sedimentación permitió obtener elevados porcentajes de eliminación, especialmente de la turbidez, CI, DQO, CFTs y NT, con porcentajes de eliminación igual al 92,5%, 90,2%, 57,5%, 98,7% y 52,8%, respectivamente.
- La microfiltración permitió porcentajes de eliminación del 98%, 82,6%, 13,8%, 85,1% y 22,7% para la turbidez, DQO, CFTs, COT y NT, respectivamente.
- En cuanto a la eliminación de compuestos fenólicos, la floculación-sedimentación demostró ser la etapa más efectiva.

5.2.3. Cultivo de *Scenedesmus obliquus* en las aguas residuales de almazara pretratadas y características bioquímicas de la biomasa final.

- La velocidad específica máxima de crecimiento registró un aumento a concentraciones bajas de ARA (μ_m = 0,035 h^{-1} in 5% OMW) y una disminución (μ_m = 0,0232 h^{-1} in 100% OMW) a concentraciones ≥ 50%.
- En cuanto a la productividad de biomasa, el mayor valor registrado fue igual a 0,896 mg/(L h) en el medio de cultivo con 100% ARA. Esto se debe a la mayor concentración de nitrógeno total en el medio.
- La concentración final de biomasa obtenida varió entre 0,029 g/L (5% OMW) y 0,21 g/L (100% OMW). A pesar de ser unos valores bajos, el objetivo principal del bioproceso es el tratamiento de las ARA y simultáneamente, la producción de biomasa con alto valor añadido.
- La biomasa final registró valores de hasta el 72,5% de carbohidratos y 44,9% de lípidos en los medios de cultivo con 5% y 100% de ARA, respectivamente. Hecho que se debe a la acumulación de compuestos energéticos bajo condiciones de estrés ambiental. En cuanto

al contenido en proteínas, el mayor valor registrado fue igual a 64,2%, en el medio de cultivo con 50% ARA.

- Los perfiles de ácidos grasos revelaron que la concentración de estos está influenciada por la composición del medio de cultivo y la intensidad de luz recibida por el cultivo. Los ácidos grasos saturados fueron los más abundantes (51,1-64,1%) y dentro de este grupo el ácido palmítico (42,3%-54,8%) y el esteárico (6,18%-7,10%) se encontraron en mayor concentración.

5.2.4. Efecto del cultivo de *Scenedesmus obliquus* en las características fisicoquímicas de las aguas residuales de almazara.

- Los mayores niveles de eliminación de carbón orgánico (67,4%) e inorgánico (95,8%) se registraron en el medio de cultivo con 50% ARA, correspondiéndose el mayor descenso en su concentración con la fase exponencial de crecimiento.
- Mayores porcentajes de eliminación de nitrógeno (98,2% en los cultivos con 50% y 75% ARA) dieron lugar a una biomasa final con un mayor contenido en proteínas (64,2% y 55,4% respectivamente).
- En cuanto a la eliminación de compuestos fenólicos, las máximas velocidades (-1,06 μg/(L h) and -1,60 μg/(L h)) y porcentajes de eliminación (54,4% y 59,1%) se determinaron en los medios de cultivo con 5% y 10% de ARA, respectivamente.
- En base a las concentraciones finales de los compuestos fenólicos, todas las ARAs tratadas podrían ser directamente descargadas en el alcantarillado público, con un límite permisible máximo de 5 mg/L. Además, aquellos cultivos con ARA ≤ 50% pueden ser descargados en aguas superficiales, con un límite permisible de 1 mg/L.

5.3. Cultivo de *Scenedesmus obliquus* en mezclas de aguas residuales urbanas y aguas de almazara para la producción de biomasa microalgal y el tratamiento de las aguas residuales.

5.3.1. Características fisicoquímicas de las aguas residuales.

- Las aguas residuales crudas de almazara utilizadas se caracterizaron por un elevado contenido en materia orgánica, determinada mediante los siguientes valores: turbidez = 714 FTU, DQO = 5839 mg O_2/L, CFTs = 322 mg/L, COT = 646 mg/L y NT = 58,9 mg/L. Dichos valores se han reducido tras una floculación-sedimentación por Flocudex CS-51 y un tratamiento por luz UV (durante 30 min) a turbidez = 32,9 FTU, DQO = 3746 mg O_2/L, CFTs = 21 mg/L, COT = 372 mg/L y NT = 6,56 mg/L.
- El agua residual urbana (ARU) cruda empleada en la realización de mezclas presentó un elevado contenido en cloro (Cl^- = 202 mg/L) y sulfato (SO_4^{-2} = 579 mg/L), ambos compuestos provocan inhibición del crecimiento a elevadas concentraciones. Sin embargo, su contenido en materia orgánica (turbidez = 26,3 FTU, DQO = 110 mg O_2/L y COT = 22,1 mg/L y NT = 6,99 mg/L) fue menor.
- El ARU filtrada con una membrana de 0,2 μm y empleada como medio de cultivo único presentó una concentración de nitrógeno total mayor (NT = 20,8 mg/L), nutriente esencial en el crecimiento y en el metabolismo microalgal.

5.3.2. Crecimiento de *Scenedesmus obliquus* y composición bioquímica de la biomasa final obtenida.

- Los valores más altos de la velocidad específica máxima de crecimiento (μ_m = 0,0202 h^{-1}) y la productividad volumétrica en biomasa (P_b = 1,21 mg/(L h) fueron obtenidos en el medio de cultivo con 100% agua residual urbana.
- El mayor contenido en proteínas se obtuvo en la biomasa final procedente del medio de cultivo constituido por 100% agua residual urbana (NT = 20,8 mg/L y 57,7% proteínas). La biomasa obtenida en los medios de cultivo constituidos por mezclas registró valores significativamente más bajos debido a la deficiencia de nitrógeno.

- La acumulación de carbohidratos se vio favorecida por las condiciones de estrés ambiental, alcanzándose valores de hasta el 75,3% de carbohidratos en el medio de cultivo formado por 10%ARA/90%ARU (v/v).
- Un comportamiento similar se observó en el porcentaje de lípidos, alcanzando el mayor valor (19,7%) en el medio de cultivo compuesto por la mezcla 5%ARA/95%ARU (v/v).

5.3.3. Características de las aguas residuales después del cultivo de *Scenedesmus obliquus*.

- La mayoría de los parámetros fisicoquímicos estudiados registraron una disminución tras el cultivo de *S. obliquus,* a excepción de la turbidez, DQO y COT, provocado por la presencia de roturas celulares al final de los cultivos.
- Los niveles más elevados de eliminación de COT y CI se registraron durante la fase exponencial de crecimiento, demostrando la capacidad de *S. obliquus* de crecer mixotróficamente asimilando compuestos orgánicos, inorgánicos y CO_2.
- Un comportamiento similar se observó para el consumo de nitrógeno, alcanzando valores de hasta el 85,4% de eliminación (100% ARU). La reducción de este nutriente en las aguas residuales es esencial para su reutilización y vertido a los cauces públicos permitiendo evitar el fenómeno de eutrofización en las aguas receptoras.
- En todos los cultivos realizados se registró una disminución en la concentración de compuestos fenólicos, alcanzándose valores de hasta el 90,8% (10%ARA/90%ARU, v/v). La eliminación de estos compuestos es especialmente importante para la reutilización de agua residuales en actividades de riego o para su vertido en aguas receptoras.

5.4. Determinación de la estabilidad a la oxidación térmica y de los parámetros cinéticos del aceite de oliva virgen extra de diferentes variedades

5.4.1. Perfil de ácidos grasos de los aceites de oliva virgen extra de diferentes variedades

- La importancia de los perfiles de ácidos grasos de los aceites de oliva reside en el hecho de que identifican la estabilidad de dichos aceites frente a la oxidación, además de que son indicadores de su alta calidad para el consumo humano.
- Las diferencias observadas en el perfil de ácidos grasos de las cuatro variedades de aceite de oliva virgen extra (AOVE) estudiadas se deben a factores agronómicos, ambientales, de procesamiento, climáticos, etc.
- El ácido graso más abundante fue el ácido oleico (C18:1 n9) con un contenido promedio del 77,1%, seguido del ácido palmítico (C16:0) con una concentración media del 11,5%. Les siguieron el ácido linoleico (C18:2 = 6,44%), esteárico (C18:0 = 1,99%) y linolénico (C18:3 = 1,29%).
- Los ácidos grasos saturados constituyeron el 13,6% del total, mientras que los monoinsaturados y poliinsaturados representaron el 77,4% y el 8,98%, respectivamente. El total de ácidos grasos insaturados constituyó el 86,4%.

5.4.2. Calorimetría Diferencial de Barrido.

- Las cuatro variedades de AOVE mostraron un comportamiento similar al ser sometidas a un proceso de termo-oxidación no isotérmica. En todos los casos se observó la aparición de un pico correspondiente al punto en el que comienza la termo-oxidación (temperatura de inicio de la oxidación).
- Mayores velocidades de calentamiento dieron lugar a mayores valores en la temperatura de inicio de la oxidación.
- El tiempo de inducción a la oxidación fue estimado para dos temperaturas representativas: 25ºC y 150ºC. Los resultados demostraron que a 150ºC, los aceites de las cuatro variedades

tienen una estabilidad termo-oxidativa a alta temperatura muy similar, siendo el aceite procedente de la variedad Arbequina el menos estable.

- Los resultados a 25ºC también revelaron que el aceite de la variedad Arbequina es el menos estable, teniendo el aceite de las variedades Coupage Changlot Real y Arbosana una vida útil cuatro veces mayor. Sin embargo, puesto que la temperatura de 25ºC se encuentra más lejos del rango experimental estudiado, tanto las diferencias en los valores de este parámetro como las barras de error obtenidas fueron más significativas.
- La temperatura de 25°C se encuentra muy lejos del rango experimental y la extrapolación no lineal afecta tanto a la exactitud como a la precisión de los resultados.
- Las diferencias obtenidas en la estabilidad oxidativa de los cuatro aceites de oliva de las cuatro variedades están directamente relacionadas con el perfil de los ácidos grasos de los mismos. Los procesos de oxidación ocurren principalmente en dobles enlaces, por lo que los ácidos grasos con mayor número de insaturaciones son menos estables y más propensos a la oxidación.
- La Calorimetría Diferencial de Barrido es una técnica eficiente, rápida y precisa para la evaluación de la calidad y estabilidad del aceite de oliva.

5.4.3. Espectrofotometría ultravioleta

- Los cuatro aceites de oliva de las cuatro variedades mostraron espectros similares tanto en el rango UV como en el visible.
- Los cuatro aceites de oliva estudiados cumplen con el criterio establecido por el Consejo Oleícola Internacional y el Reglamento de la Comisión Europea puesto que los valores de K_{232} y K_{270} fueron menores que los límites establecidos (2,50 y 0,22, respectivamente).
- Los resultados indicaron la ausencia de productos tanto primarios como secundarios derivados de la oxidación de los aceites de oliva.

CONCLUSIONS

The following conclusions have been obtained after the interpretation and discussion of the experimental results. Considering the nature of this Doctoral Thesis, which is presented in the form of a compendium of already published articles, the conclusions will be divided into articles:

5.1. Integrated process for olive oil mil wastewater treatment and its revalorization through the generation of high added value algal biomass.

5.1.1. Physicochemical characteristics of raw olive oil mill wastewaters.

- The olive oil mill wastewaters (OMW) were characterized by a high content in organic matter, determined in terms of turbidity = 714 FTU, chemical oxygen demand (COD= 5839 mg O_2/L), total phenolic compounds (TPCs = 322 mg/L), total organic carbon (TOC = 646 mg/L) and total nitrogen (TN = 58.9 mg/L). OMW has a high chemical oxygen demand, rich in natural antioxidant (growth inhibitory compounds), which are difficult to be biodegraded.
- OMW registered a high concentration of phenolic compounds (TPCs = 322 mg/L). Their structure, high specific chemical oxygen demand, phytotoxicity and antibacterial activity make them difficult to be biodegraded and contribute to the high toxicity of OMW.
- The high concentration of inorganic salts (inorganic carbon = 318 mg/L) in OMW as well as phosphorous content (ortho-phosphate = 43.1 mg/L) promoted microalgal growth due to their role in the metabolism of microalgae.
- The high COD/TOC value registered (equal to 9) is explained by the high heterogeneity of industrial OMW.

5.1.2. Effect of primary treatment (flocculation-sedimentation, UV photolysis and membrane microfiltration) on OMW physicochemical characteristics.

- Primary treatment allowed high organic matter removal with percentages up to 96.2%, 80.3% and 96.6% for COD, TOC and TPCs, respectively.

- Flocculation-sedimentation proved to be the most effective operation in phenols removal, with an elimination percentage of 78% compared to the 45.7% and 72.7% achieved after UV photolysis and microfiltration, respectively.
- Regarding organic matter removal, UV photolysis proved the most effective operation. Removal percentages registered after flocculation, UV photolysis and microfiltration were 57.7%, 88.8% and 20.5% for COD and 18%, 71.9% and 14.6% for TOC, respectively.

5.1.3. *Chlorella pyrenoidosa* growth in pretreated OMW and biochemical composition of the final biomass.

- The highest values of the maximum specific growth rate ($\mu_m = 0.07\ h^{-1}$) and volumetric biomass production (P_b = 1.25 mg/(L h)) were achieved in the culture with 50% OMW (v/v).
- For both kinetic parameters (μ_m y P_b), an increase of their values with the rise of OMW concentration in the culture medium until 50% OMW (v/v) were registered. Then, both parameters were decreased due to the toxic or inhibitory effect of OMW.
- The final biomass was characterized by a high content of carbohydrates, with values that varied from 30.3% to 89.2% for cultures with 100% and 5% OMW (v/v), respectively. This fact is explained by the ability of microalgae to accumulate energetic compounds under environmental stress conditions.
- The protein content in final biomass was increased with the rise of OMW concentration reaching a maximum value of 51.5% in 100% of OMW culture medium, explained by the higher nitrogen availability.
- The highest lipid content (34.2%) was registered in the culture with 25% OMW (v/v). Regarding the profiles of fatty acids in the lipid fraction, saturated were the most abundant (85.2-95.1%) where palmitic acid varied from 65.7% to 74.7%.

5.1.4. Secondary treatment (*Chlorella pyrenoidosa* culture) effect on OMW physicochemical characteristics.

- Higher removal percentages were obtained in more diluted culture media, explained by a lower concentration of inhibitory compounds in OMW media. Removal percentages of up to 74%, 75.5%, 71.3% and 87.6% were obtained for TC, TOC, IC and TN in the culture medium of 25% of OMW (v/v), respectively.
- Experimental results revealed that removal rates of organic matter (TOC and TN) and inorganic carbon were sharply increased during the exponential growth phase, which is explained by the ability of *C. pyrenoidosa* to grow mixotrophically assimilating organic and inorganic compounds as carbon source.
- The highest TPCs removal value (67.1%) was registered in the culture medium with 50% OMW (v/v), being decreased at higher OMW concentrations due to inhibitory effect.
- At the end of the bioprocess, high quality treated waters were obtained with physicochemical characteristics that make them suitable for reuse in irrigation, industrial activities or discharge into receiving waters.

5.2. Combination of physicochemical operations and algal culture as a new bioprocess for olive mill wastewater treatment.

5.2.1. Physicochemical characterization of raw olive mill wastewater

- Raw OMW registered a high organic matter content, the main parameter to consider from the environmental point of view, and determined in terms of turbidity = 714 FTU, COD = 5839 mg/L, TPCs = 322 mg/L, TOC = 328 mg/L and TN = 58.9 mg/L.
- The content of total nitrogen (TN = 58.9 mg/L) and phosphate (PO_4^{-3} = 43.1 mg/L) registered in raw OMW was notably lower than that contained in the control synthetic medium of Rodríguez-López, with 140 mg/L and 160 mg/L, respectively.

5.2.2. Primary treatment (flocculation-sedimentation and membrane filtration) effect on the physicochemical characteristics of olive mill wastewater

- Primary treatment proved to be effective in the reduction of the most of parameters studied, especially in the elimination of total solids, which resulted in the decrease of inhibitory compounds, turbidity and color.
- High elimination percentages were obtained through flocculation-sedimentation, especially in turbidity, IC, COD, TPCS and TN, with removal percentages values equal to 92.5%, 90.2%, 57.5%, 98.7% and 52.8%, respectively.
- The microfiltration allowed removal percentages of 98%, 82.6%, 13.8%, 85.1% y 22.7% for turbidity, COD, TPCs, TOC and TN, respectively.
- Flocculation could be highlighted as the most effective stage in terms of phenolic compounds elimination. This is especially important due to the inhibitory effect of phenolic compounds in microalgal growth.

5.2.3. *Scenedesmus obliquus* culture in pretreated olive oil wastewaters and biochemical composition of the final biomass.

- Maximum specific growth rate registered the highest value $\mu_m = 0.035\ h^{-1}$ in 5% OMW and decreased to $\mu_m = 0.0232\ h^{-1}$ in 100% OMW (lower values for μ_m in cultures with OMW concentrations $\geq$ 50%).
- The highest biomass productivity value was equal to 0.896 mg/(L h) and registered in the culture with 100% of OMW. This fact can be explained by the higher nitrogen concentration in this medium.
- The final biomass concentration at the end of the cultures ranged from 0.029 g/L (5% OMW) to 0.21 g/L (100% OMW). Although these concentrations are low, the main goal of the bioprocess is the OMW treatment and simultaneously, the production of algal biomass with high added value.
- In the culture media with 5% and 100% of OMW the harvested biomass had 72.5% of carbohydrates and 44.9% of lipids, respectively. This fact can be explained by the

accumulation of energetic compounds under environmental stress conditions. Regarding the protein content, the highest value was equal to 64.2% in the culture with 50% of OMW.

- The fatty acid profiles revealed that fatty acids concentration is influenced by the culture medium composition and the light intensity received by the culture. Saturated fatty acids were the most abundant (51.1%-64.1%) and within this group, palmitic (42.3%-54.8%) and stearic acids (6.18%-7.10%) were found at higher concentrations.

5.2.4. Effect of *Scenedesmus obliquus* culture in olive oil mill wastewater physicochemical characteristics.

- The highest removal values for TOC (67.4%) and IC (95.8%) were registered in the culture with 50% of OMW, corresponding the sharpest decline in their concentrations with the exponential growth phase.
- Higher nitrogen removal values equal to 98.2% in cultures with 50% and 75% of OMW resulted in a final biomass with a higher protein content of 64.2% and 55.4%, respectively.
- For phenolic compounds removal, the highest removal velocities (-1.06 and -1.60 μg/(L h)) and percentages (54.4% and 59.1%) were determined in the culture media with 5% and 10% of OMW, respectively.
- Based on the final TPCs concentration, all treated OMW could be directly discharged into public sewers, with a permissible limit of phenols equal to 5 mg/L. Furthermore, cultures with OMW ≤ 50% are suitable for discharge into inland surface waters, with an admissible limit of 1 mg/L.

5.3. Cultivation of *Scenedesmus obliquus* in mixtures of urban and olive-oil mill wastewaters for the dual application of algal biomass production and wastewater treatment.

5.3.1. Wastewaters physicochemical characteristics.

- Raw olive oil mill wastewater was characterized by a high organic matter content, defined by turbidity = 714 FTU, COD = 5839 mg O_2/L, TPCs = 322 mg/L and TOC = 646 mg/L and TN = 58.9 mg/L. These values decreased after flocculation-sedimentation using

Flocudex CS-51 and treatment by UV (during 30 min) to turbidity = 32.9 FTU, COD = 3746 mg O_2/L, TPCs = 21 mg/L, TOC = 372 mg/L and TN = 6.56 mg/L.

- Raw urban wastewater (UW) used in the mixtures with OMW, registered a high chloride (Cl^- = 202 mg/L) and sulphate concentration (SO_4^{-2} = 579 mg/L), both inhibitory compounds at high concentrations. Nevertheless, the organic matter content (turbidity = 26.3 FTU, COD = 110 mg O_2/L, TOC = 22.1 mg/L and TN = 6.99 mg/L) was significantly lower in comparison with OMW.
- Filtered urban wastewater (by membrane 0.2 μm) used as single culture medium showed the highest nitrogen concentration (TN = 20.8 mg/L), an essential nutrient for microalgae growth.

5.3.2. *Scenedesmus obliquus* growth and biochemical composition of the final biomass.

- The highest values of maximum specific growth rate (μ_m = 0.02 h^{-1}) and volumetric biomass productivity (P_b = 1.21 mg/(L h)) were obtained in the culture with 100%UW.
- The highest protein content was registered in the biomass obtained from the 100% of UW culture (TN = 20.8 mg/L and 57.7% proteins). Biomass from culture media formed by mixtures registered significantly lower values due to nitrogen deficiency.
- Carbohydrates accumulation was favored by environmental stress conditions, reaching values up to 75.3% in the culture medium formed by 10% of OMW and 90% of UW (v/v).
- Similar behavior was observed in the lipid content, which reached the highest value (19.7%) in the culture medium formed by 5%OMW/95%UW (v/v).

5.3.3. Wastewaters physicochemical characteristics after *Scenedemsus obliquus* culture.

- Most physicochemical parameters studied showed a decrease after *S. obliquus* culture, except turbidity, COD and TOC due to the presence of cell ruptures in the treated wastewater after biomass separation by centrifugation.

- The highest TOC and IC removal levels were registered during the exponential growth phase, which shows the ability of S. *obliquus* to grow mixotrophically assimilating organic and inorganic (CO_2) compounds and as carbon source.
- Similar behaviour was observed in the consumption of nitrogen, reaching removal values of up to 85.4% (in culture with 100% UW). The reduction of this nutrient in wastewater is highly relevant to avoid the eutrophication of receiving waters.
- Phenols concentration was decreased in all experiments, reaching removal values up to 90.8% (in culture with 10%OMW/90%UW). The removal of these compounds is especially important for the reuse of wastewaters in irrigation or for its discharge into receiving waters.

5.4. Determination of the thermal oxidation stability and the kinetic parameters of commercial extra virgin olive oils from different varieties.

5.4.1. Fatty Acids composition of different extra virgin olive oils varieties.

- The importance of olive oil fatty acids (FAs) profile is because high- and poor-quality olive oils differ in their content in metabolites derived from oxidation reactions of certain fatty acids, being linoleic and linolenic the main substrates.
- The differences observed in the FAs profile of the four extra virgin olive oils (EVOO) studied may be due to several factors such as agronomic, climatic, environmental, or processing factors.
- The most abundant FAs was oleic acid, with an average content of 77.1%, followed by palmitic acid (11.5% on average), linoleic (6.44%), stearic (1.99%) and linolenic (1.29%).
- Saturated fatty acids constituted the 13.6% of the total FAs, followed by the monounsaturated and polyunsaturated fatty acids, which comprised 77.4% and 8.98%, respectively. Total unsaturated FA represented 86.4%.
- The fatty acid profile constitutes an indicator of olive oils nutritional quality.

5.4.2. Differential Scanning Calorimetry.

- The four EVOO studied showed a similar behavior when subjected to a non-isothermal thermo-oxidation process. The appearance of a peak corresponding to the start of thermal oxidation was observed in all cases (oxidation onset temperature).
- Results showed that higher heating rates lead to higher oxidation onset temperatures (OOT).
- The oxidation induction time (OIT) was predicted for two representative temperatures: 25ºC and 150ºC. Results showed that at 150ºC, all varieties have a similar high-temperature thermo-oxidative stability, with Arbequina being the least stable.
- The results at 25ºC also suggested that Arbequina is the least stable variety, having the Coupage Changlot Real and Arbosana about four times longer shelf life. However, since 25ºC is farther away from the studied experimental range, the differences in OIT values as well as the error bars were more significant.
- The temperature of 25ºC lies far away from the experimental range, affecting both the accuracy and precision of the results.
- The differences obtained in the oxidative stability of the four EVOO varieties are related to their FAs profiles. Oxidation processes occur mainly in double bonds, so FAs with higher unsaturation are less stable and more prone to oxidation.
- Differential Scanning Calorimetry is an efficient, fast and precise technique for the evaluation of olive oil quality and stability.

5.4.3. Ultraviolet Spectrophotometry

- The four EVOO of different varieties showed similar spectra both in the UV and in the visible range.
- The four EVOO of different varieties studied meet the criteria established for EVOO by the International Olive Oil Council and the Commission Regulation since K_{232} and K_{270} values were lower than the established limits (2.50 and 0.22, respectively).

- Results proved the absence of primary and secondary products derived from olive oil oxidation.

6. PUBLISHED ARTICLES

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Integrated process for olive oil mill wastewater treatment and its revalorization through the generation of high added value algal biomass

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ABSTRACT

The two-phase continuous centrifugation process for olive oil extraction generates high amounts of olive oil mill wastewater (OMW), characterized by containing large concentrations of numerous contaminant compounds for the environment. An integral process based on physico-chemical (flocculation, photolysis and microfiltration) and microalgal growth stages was proposed for its treatment. Chemical oxygen demand (COD) removal percentages were 57.5%, 88.8% and 20.5% for flocculation, photolysis and microfiltration, respectively. The global removal percentages of organic load in the primary treatment were 96.2% for COD, 80.3% for total organic carbon (TOC) and 96.6% for total phenolic compounds (TPCs). In secondary treatment, different experiments using the microalgae *Chlorella pyrenoidosa* were performed on a laboratory scale in stirred batch tank reactors. The OMW concentrations in each culture medium were: 5%, 10%, 25%, 50%, 75% and 100% (v/v). The common experimental conditions were: pH = 7, temperature = 25 °C, agitation speed = 200 rpm, aeration rate = 0.5 (v/v) and illumination intensity = 359 μE m^{-2} s^{-1}. The highest maximum specific growth rate (0.07 h^{-1}) and volumetric biomass production (1.25 mg/(L h)) values were achieved in the culture with 50% of OMW (v/v). The final biomass obtained had a high percentage of carbohydrates, whose content ranged from 30.3% to 89.2% and the highest lipid content (34.2%) was determined in the culture with 25% of OMW (v/v). The final treated water is suitable for its use in irrigation, discharge to receiving waters or for being reused in the same process.

1. Introduction

Microalgae are photosynthetic microorganisms that are characterized by its easy culture, and high growth and productivity rates. These microorganisms produce biomass with high-added value products as pharmaceutical compounds, fatty acids, carotenoids, dyes and fine chemicals. All these compounds can be used for human, animal and aquatic feed (Hodaifa et al., 2013; Mata et al., 2010; Nor et al., 2016; Rawat et al., 2011; Suganya et al., 2016). On the other hand, they are able to grow in harsh conditions requiring water, inorganic salts, CO_2 and sunlight (Mata et al., 2010). In this sense, microalgae have numerous environmental applications such as CO_2 mitigation and wastewater treatment (Suganya et al., 2016). Furthermore, certain species have the capacity to degrade a large variety of compounds such as xenobiotic, polyaromatic hydrocarbons, phenolic compounds, pesticides, etc. For all these reasons, the dual application of microalgae for wastewater treatment and biomass production is an attractive alternative with great industrial and economic potential (Hodaifa et al., 2012; Rawat et al., 2011).

Different wastewaters such as municipal, agricultural and piggery have been used as microalgae culture media for nutrient removal and biomass production (Abou-Shanab et al., 2013; Ji et al., 2014; Mata et al., 2010; Rawat et al., 2011). Many works have shown the ability of microalgae to degrade and remove excess nutrients (mainly persistent and hazardous organic pollutants) in

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wastewaters. The capacity of *Chlorella, Ankistrodesmus* and *Scenedesmus* species to remove contaminants from olive oil mill and paper industry wastewaters has already been demonstrated (Hodaifa et al., 2012, 2013; Kouhia et al., 2015). In general, wastewaters have a complex physicochemical composition, for this reason, the nutrient availability and the presence of growth inhibitors could influence microalgal growth (Guldhe et al., 2017; Hodaifa et al., 2012).

Olive oil industry is an important sector within the agro-food industries in the Mediterranean countries (Hodaifa et al., 2012) as well as in non-traditional producing countries (as Australia, New Zealand and South America) due to the growing interest in olive oil consumption and production. Olive oil is obtained from olive fruit by mechanical procedures throughout pressing (discontinuous) and centrifugation systems (continuous). The last systems can be carried out by using a 'Decanter' with two or three exits (Dermeche et al., 2013). In Spain, the main olive oil producer worldwide, the centrifugation process using a 'Decanter' with two exits (for olive oil and pomace production) is currently used (Tsagaraki et al., 2007). Olive oil mill wastewater (OMW) from two exits is characterized by containing a high concentration of organic matter which includes polysaccharides, sugars, phenolic compounds, polyalcohol, nitrogenous compounds, organic acids, tannins, pectin, lignin, oil and high levels of suspended solids (Dermeche et al., 2013; Mantzavinos and Kalogerakis, 2005). In this sense, OMW produced by 'Decanter' with two exits have less organic load (COD = 4–16 g O_2/L) in comparison with the wastewaters generated using a 'Decanter' with three exits or the pressing process (COD = 40–220 g O_2/L), (Agabo-García and Hodaifa, 2017).

In this work, a new process for real OMW treatment based on physico-chemical operations (as primary treatment) followed by microalgae culture (as secondary treatment) was proposed. First operations included flocculation-sedimentation, photolysis and microfiltration units connected with *Chlorella pyrenoidosa* culture. In this sense, physico-chemical characteristics of the real crude olive oil mill wastewater were studied. Flocculation-sedimentation and photolysis operations were established and optimized. Then, different dilutions of primary treated OMW (5%, 10%, 25%, 50%, 75% and 100% v/v) were used as culture media. Kinetic growth, biomass production and biochemical composition of *C. pyrenoidosa* were evaluated. Treated water and bioremediation of the wastewater during the integral process were determined.

2. Experimental

2.1. Microorganism and photobioreactor

The microorganism used was the freshwater green algae *Chlorella pyrenoidosa* Chich 8H Emerson. Experiments were performed in sterile conditions, on a laboratory scale in stirred batch tank reactors with work capacity = 1 L, diameter = 10 cm and height = 16 cm. All bioreactors had continuous illumination on one side.

2.2. Procedure

OMW was obtained from an olive oil extraction plant in the province of Seville (Spain). The flocculation-sedimentation was carried out during 90 min in Imhoff funnel using a commercial flocculant Flocudex CS-51. Based on a previous study (Hodaifa et al., 2015) an optimal flocculant concentration of 1 g/L was selected.

The obtained supernatant was subjected to photolysis in a batch stirred photoreactor with total capacity equal to 750 cm^3 (work volume = 600 cm^3). A commercial medium pressure UV immersion lamp, model TQ 150 Brand HNG Germany G4, 150 N° 5600 1725 (Standard) was used. During the proposed process the reduction of organic matter was determined.

Culture media were prepared by mixing OMW and ultrapure water to obtain the following final concentrations: 5%, 10%, 25%, 50%, 75% and 100% (v/v) OMW. Sterilization was performed by filtration through a membrane with pore size equal to 0.2 μm.

The pH was adjusted and maintained at a value of 7.0 over the course of the culture through the addition of 0.1 mol NaOH L^{-1} or 0.1 mol HCl L^{-1} solution.

The common culture conditions were: temperature = 25 °C, aeration rate = 0.5 L/min, pH value = 7, magnetic agitation speed = 200 rpm and continuous light with illumination intensity equal to 359 μE m^{-2} s^{-1}.

In all the experiments, the precultures of *C. pyrenoidosa* were grown for 7 day at room temperature in solidified Rodríguez-López medium (Rodríguez-López, 1964) with agar at 2% (w/w) under continuous illumination. The liquid inoculum (0.0141 ± 0.00791 g/L) for each experiment consisted of a suspension of cells in sterile Rodríguez-López culture medium.

2.2.1. Microalgae growth

The biomass concentration, x g L^{-1}, was measured indirectly by the absorbance of the cell suspension in ultrapure water at 600 nm (Camacho et al., 1989) after two centrifugation stages in which biomass was washed with ultrapure water. Results obtained allowed the representation of growth curves and the determination of the growth kinetic velocities.

The specific growth rate ($\mu = 1/x \cdot dx/dt$) in the exponential phase and the biomass productivity ($P_b = dx/dt$) in the linear phase were determined.

2.2.2. Biochemical composition of the biomass

In all experiments, the total pigments (total chlorophylls and total carotenoids) were determined during the course of the cultures. At the end of each experiment, algal biomass was separated and total lipids, proteins and fatty-acids contents were determined.

Total lipids were obtained by using a micro-soxhlet extractor with n-hexane as solvent. Fatty acid profile was determined and identified directly from dried algal biomass by gas chromatography using a Hewlett–Packard, Model 5890 Series II equipped by a FID detector (Lepage and Roy, 1984). The crude protein content was performed from the nitrogen percentage determination (%Crude proteins = %TN × 6.25, Becker, 1994) using a Total Carbon and Nitrogen Analyser provided by Skalar Company, mod. Formacs^{HT} and Formacs^{TN}.

The total carbohydrate content was obtained by considering that algal biomass is formed by proteins, carbohydrates, lipids, pigments and genetic material. For carbohydrate content calculation, genetic material was considered approximately about 1% (Becker, 1994).

2.3. Analytical methods

In the characterization of wastewater and treated water (crude and after each treatment), the following parameters were determined: pH value, electric conductivity, turbidity, chemical oxygen demand (COD), total phenolic compounds (TPCs), total organic carbon (TOC), total carbon (TC), inorganic carbon (IC), total nitrogen (TN), total iron, sulphates, sodium, *ortho*-phosphate and ammonium.

pH, electric conductivity (EC) and turbidity values were directly measured by using a pH-meter Crison, mod. GLP 22C, Conductimeter Crison, mod. GLP31 and Turbidimeter Hanna, mod. HI93703, respectively.

The determination of TPCs was carried out by making it react

with a derivative thiazol, giving a purple azo dye, which was determined photometrically at 475 nm according to the standard methods (ISO 8466-1; DIN 38402 A51).

COD was determined photometrically at 620 nm according to German standard methods (DIN 38409 H41).

TOC, TC, IC and TN contents were determined using a Total Carbon and Nitrogen Analyser provided by Skalar Company, mod. Formacs^{HT} and Formacs^{TN}.

Total iron ions determination was performed through the reduction of all iron ions to iron (II) ions in a thioglycolate medium with a derivative of triazine. This reaction results in a reddish-purple complex that was determined photometrically at 565 nm according to the standard methods (ISO 8466-1; DIN 38402 A51).

Sulphates and ortho-phosphates were determined photometrically at 420 nm and 690 nm, respectively, according to the standard methods (ISO 8466-1; DIN 38402 A51).

Sodium, ammonium, potassium and calcium contents were determined directly by using selective ion electrodes for each one (Crison, mod. GLP 22C).

Finally, carbohydrate content (total reducing sugars) could be determined by using the DNS (dinitrosalicylic acid) method as described by Miller (1959). In this method, 3 mL of DNS reagent is mixed with 2 mL of sample. Then the sample is immersed in a water bath at 80–85 °C for 5 min. After cooling to room temperature, the sample is measured photometrically at 540 nm. In addition, a calibration line using glucose as reference reagent is needed.

2.4. Calculation methods and reproducibility

In this work, experiments were made at least in duplicate and analytical methods were applied at least in triplicate. Models calculation and statistical methods used were available in the OriginPro 8.0 program.

3. Results and discussion

3.1. Characterization of raw OMW used

Wastewater must contain a suitable nutrient profile for its use as culture medium for microalgae, with carbon, nitrogen and phosphorous sources as the most essential elements required for algal biomass growth. Table 1 shows the composition of raw and treated industrial olive oil wastewater used in this work. It is necessary to highlight the high presence of high organic matter, determined in terms of turbidity = 714 FTU, COD = 5839 mg O_2/L, TPCs = 322 mg/L, TOC = 646 mg/L and TN = 58.9 mg/L. The high TN concentration registered can be explained by the presence of proteins and other nitrogenated compounds in the OMW composition, which come from the olive fruit crushing and olive oil washing (Agabo-García and Hodaifa, 2017).

High concentrations of phenols (TPCs = 322 mg/L) were also found. These latter compounds have a similar structure to that of lignin, which makes them difficult to be biodegraded. They are also characterized by a high specific chemical oxygen demand, phytotoxicity and antibacterial activity, being the major contributors to the OMW toxicity and microalgal growth inhibition (Azabou et al., 2007; D'Antuono et al., 2014; Fountoulakis et al., 2002). A high inorganic salts portion was also detected (318 mg/L), as well as phosphorus in the form of inorganic salts (*ortho*-phosphate = 43.1 mg/L), which play an important role in microalgae cell growth and metabolism through phosphorylation reactions. On the other hand, it must be also indicated the high COD/TOC ratio value (equal to 9) registered for raw OMW in comparison with domestic wastewater in which this value is around 2 to 3 (Huang et al., 2010). Similarly, high COD/TOC values have been registered in several industrial wastewater studies. Güneş et al. (2019) described industrial container and drum cleaning wastewater (Sample 3) with COD/TOC = 6.21. Agabo-García and Hodaifa (2017) determined for crude wastewater from washing olives (WOW) a COD/TOC ratio = 8.12. Buthiyappan and Abdul Raman (2019) indicated COD/TOC ratio values from 9.41 to 11.2 for textile wastewaters and Dhanke et al. (2018) established COD/TOC ratio = 24.3 for fish processing industry wastewaters. This fact can be explained by the high heterogeneity of industrial wastewaters physicochemical characteristics, which is mainly determined by the wastewater origin (Raper et al., 2018).

The low iron content can be explained by the use of drinking water in food industries for washing raw materials. High iron concentration is not desired since it is a microalgae growth inhibitor (Fazal et al., 2018).

3.2. Bioprocess for olive oil mill wastewater treatment

The proposed new real OMW treatment process was performed according to Fig. 1. The process undertaken consisted of four phases, of which, the first three phases correspond to the primary treatment and the last stage, to the secondary treatment:

Table 1
Characterization of raw and treated OMW during treatment process.

Parameter	Raw OMW	Primary treatment			Secondary treatment		
		Physico-chemical sequence treatment			%Treated OMW after algal culture (v/v)		
		Flocculated	UV	Microfiltration	25	75	100
pH	8.25	Natural[a]	Natural	Natural	7.0	7.0	7.0
Conductivity, mS/cm	1.9	1.34	1.35	1.28	0.35	0.96	1.26
Turbidity, FTU	714	53.5	21.9	2.37	6.75	14.0	14.1
COD, mg O_2/L	5839	2484	279	222	–	58.5	138
TPCs, mg/L	322	70.9	38.5	10.8	0.911	3.09	7.39
TC, mg/L	1400	561	237	199	51.8	117	153
TOC, mg/L	646	530	149	127	31.2	69.2	147
TN, mg/L	58.9	27.8	22.4	17.3	2.15	5.22	5.65
IC, mg/L	318	31.3	87.5	71.9	20.6	47.5	26.5
Iron, mg/L	1.19	1.03	0.857	0.508	0.15	0.29	0.490
Sulphate, mg/L	320	84.8	79.8	52.3	15.8	29.3	51.8
Sodium, mg/L	0.943	0.782	0.168	0.208	–	–	0.120
Ortho-phosphate, mg/L	43.1	21.7	21.3	–	–	–	–
Ammonium, mg/L	4.44	4.09	1.32	–	0.14	0.18	0.310

[a] pH *value of OMW without modifying.*

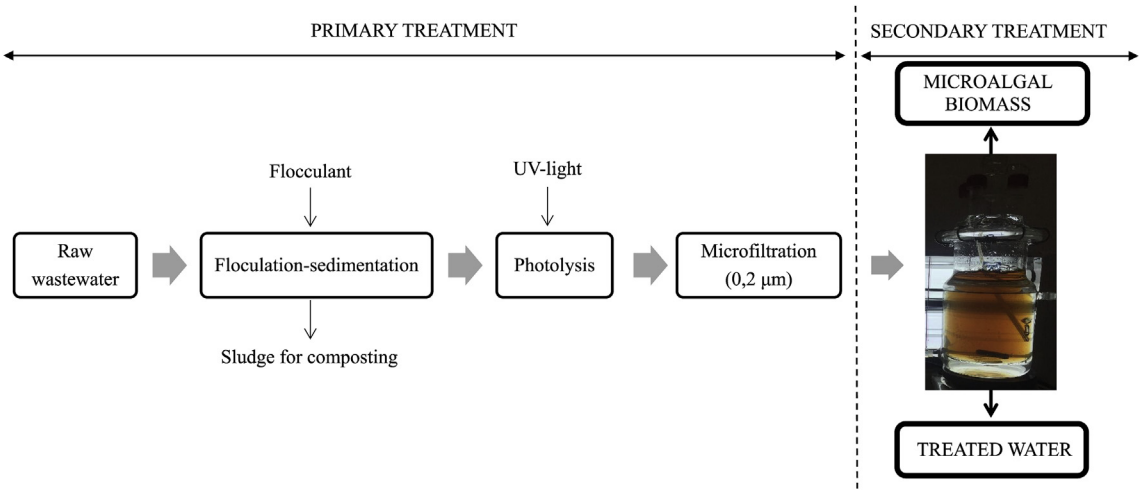

Fig. 1. Schematic representation of the new proposed bioprocess for real OMW treatment.

i) Flocculation-sedimentation. It was performed in two steps without pH modification. In the first, to complete mixing of flocculant and effluent, a high agitation speed (700 rpm) was applied for 1 min. In the second, to achieve flocs formation, a low agitation speed (350 rpm) for 30 min was employed. The aim of this stage was to separate and remove the solid fraction of OMW, which consisted of a sludge that can be used subsequently for composting. For this purpose, flocculated OMW was left to settle during 30 min.
ii) Photolysis. It consisted on the exposition of the obtained supernatant (after removal of the solid fraction) to UV-light for 30 min. The objective of this stage was the elimination of a part of the organic matter present in OMW, especially organic compounds as phenols, which are considered as microbial growth inhibitors. Sample settling during 30 min was performed to allow the sedimentation and subsequent separation of the remaining solid fraction.
iii) Microfiltration. It was used for OMW microbial (sterilization) and organic load reduction.
iv) *Chlorella pyrenoidosa* culture for the bioremediation of OMW and the obtaining of microalgal biomass with added value, mainly energetic compounds, which could be used for biofuels and biogas production or directly used in boilers for biomass combustion.

3.2.1. Primary treatment

Table 1 shows the variation of the treated water composition during the primary treatment. In general, all parameters were decreased throughout the primary treatment. Flocculation stage allowed a high total phenolic compounds removal percentage of up to 78% (Table 1). Theoretically, after the use of flocculant in OMW treatment, an increase in TPCs is expected due to the presence of phenolic compounds in the flocculant composition. The commercial Flocudex CS51 used is a solid cationic polyelectrolyte with high molecular weight and high capacity to eliminate suspended solids, turbidity and compounds responsible for colour apparition. In this sense, it is important to indicate that commercial flocculants usually incorporate a lignosulfonate, guaiacol (methoxy phenol) or protocatechuic acid in the synthesis process of acrylamide copolymers (He et al., 2015). After the photolysis operation, the TPCs concentration was decreased to 38.5 mg/L (%TPCs removal = 45.7%) due to the degradation process of lignin and phenols by the UV-light (El Hajjouji et al., 2007; Machado et al., 2000). Lignin polymer, which is largely present in olives pulp, is a natural polymer whose main structural units are phenolic compounds (Tanaka et al., 1999).

From the environmental point of view, the organic load can be determined by COD and TOC parameters. During the flocculation, photolysis and microfiltration the removal percentages 57.5%, 88.8% and 20.5% for COD and 18.0%, 71.9% and 14.6% for TOC were determined, respectively.

As a result of the flocculation process, the TOC/TN ratio increased from 11.0 (crude OMW) to 19.1 (flocculated OMW) indicating a strong fall in nitrogen content due to the efficient protein removal (component with high molecular weight) by the flocculant. After that, the ratio decreased to 6.66. This showed that during flocculation, a high percentage of proteins were removed and during photolysis, higher levels of organic matter oxidation were achieved. In general, the variation in the different determined ratios after flocculation does not follow a fixed pattern. In this sense, COD/TOC ratio was decreased from 9.04 to 4.69 through flocculation. This separation depends on the aggregation mechanism applied (charge neutralization, entrapment mainly by Van der Waals forces, adsorption forces, complexation with coagulant metal/flocculent ions into insoluble particulate aggregates, Matilainen et al., 2010). Therefore, the separation mechanism through flocculation is a non-selective separation.

During microfiltration the TOC/TN ratio registered a slightly increase (7.4) indicating higher carbon compounds removal in comparison with the elimination of nitrogenated compounds.

In view of the results achieved, it can be confirmed that photolysis was the most effective operation for organic load reduction. Von Sonntag (2008) showed the effectiveness of UV-light for organic matter photodegradation in comparison with natural oxidation. Photolysis is a photochemical operation in which organic compounds are partially decomposed because of the absorption of this high-energy irradiation. Agabo-García and Hodaifa (2017) studied the UV-light effect in the degradation of OMW organic matter in photoreactors. They observed that photodegradation occurs in one step by an instantaneous reaction in the first minutes (<4 min). Afterwards, no significant degradation was observed. In addition, Catalá et al. (2015) when using a 150 W medium pressure mercury lamp (The same UV-lamp used in this work) in natural fluvial waters containing illicit drugs achieved high TOC removal level equal to 79%.

This high elimination percentage obtained after photolysis is due to the special characteristics of UV-lamp used, wide emission

range and high potency. In this case, a commercial medium pressure UV immersion lamp, model TQ 150 Brand HNG Germany G4, 150 W, N° 5600 1725 (Standard) was used. In general, medium pressure mercury lamps are available in different potency from 100 to 1000W. The emission profile of these lamps consists on a wide range of wavelengths from 200 to 700 nm (UV and visible light) and the peak of 254 nm is strongly diminished. The emission intensity of these lamps is at least 10 fold higher than that of low-pressure arcs but happens on a much smaller surface. This UV-lamp type in contrast to other develops a considerable amount of heat, which cooling is required, but this problem can be resolved by running tap water to maintain the temperature around 20 °C (Albini and Germani, 2010). In addition, this fact is not important when working at pilot or industrial plant since the reactor volume itself is enough to remove the heat generated by the UV-lamp.

Other authors have shown that artificial UV-light oxidation allows the rapid decomposition of toxic compounds such as nitrosodimetylamine (NDMA), hydrazine, 1,4-dioxane and methylthrethylbutaneethyl (MTBE), (McCurry et al., 2016; Radjenovic et al., 2012; Tawabini et al., 2013).

Sulphate ions were efficiently removed during the primary treatment (Table 1). High sulphate ions removal percentages (73.7% and 34.5%) were registered after flocculation and microfiltration, respectively. Sulphate ions elimination from water and wastewater is complex due to the high solubility and stability of these anions in aqueous solutions. The main methods used for its treatment are: (1) biological degradation, (2) membrane filtration (primarily reverse osmosis), (3) adsorption/ion exchange in resins, and (4) chemical precipitation (Amaral Filho et al., 2016).

3.3. Secondary treatment (microalgal treatment)

3.3.1. Chlorella pyrenoidosa growth

Fig. 2A shows a sample of the growth curves of *C. pyrenoidosa* when the microalgae was grown in a 10% OMW (v/v) culture. In general, a short duration (<18 h) lag or adaptation phase was detected in all experiments. This phase was followed by an exponential growth phase whose duration ranged from 20 to 32 h in the cultures with %OMW<75% (v/v). Only in the case of 100% OMW (v/v) the duration of this phase was 61.5 h. Then, a deceleration growth phase with linear behaviour was observed. The duration of the linear growth was increased with the augment of %OMW in the culture medium (from 25 to 144 h). This appears to indicate that this phase is determined by the limitation of one or more nutrients. A stationary phase of growth at the end of the culture was observed in all experiments. In this sense, similar growth curves were obtained by Hodaifa et al. (2008, 2009, 2012) using OMW from two and three-phase systems as culture media for *Scenedesmus obliquus*.

The determination of the maximum specific growth rate and biomass productivity of *C. pyrenoidosa* were determined according equations (1) and (2), respectively (Fig. 2A).

$$ln\left(\frac{x}{x_o}\right) = \mu_m\, t + a \tag{1}$$

where 'μ_m' is the slope of the line and corresponds to the maximum specific growth rate and 'a' is the intercept.

$$x = P_b\, t + b \tag{2}$$

where 'P_b' is the slope of line and corresponds to the volumetric biomass productivity and 'b' is the intercept.

It can be observed in Fig. 3 the variation of the maximum specific growth rates (μ_m) and the biomass productivities (P_b) when initial OMW concentrations were increased in the culture medium.

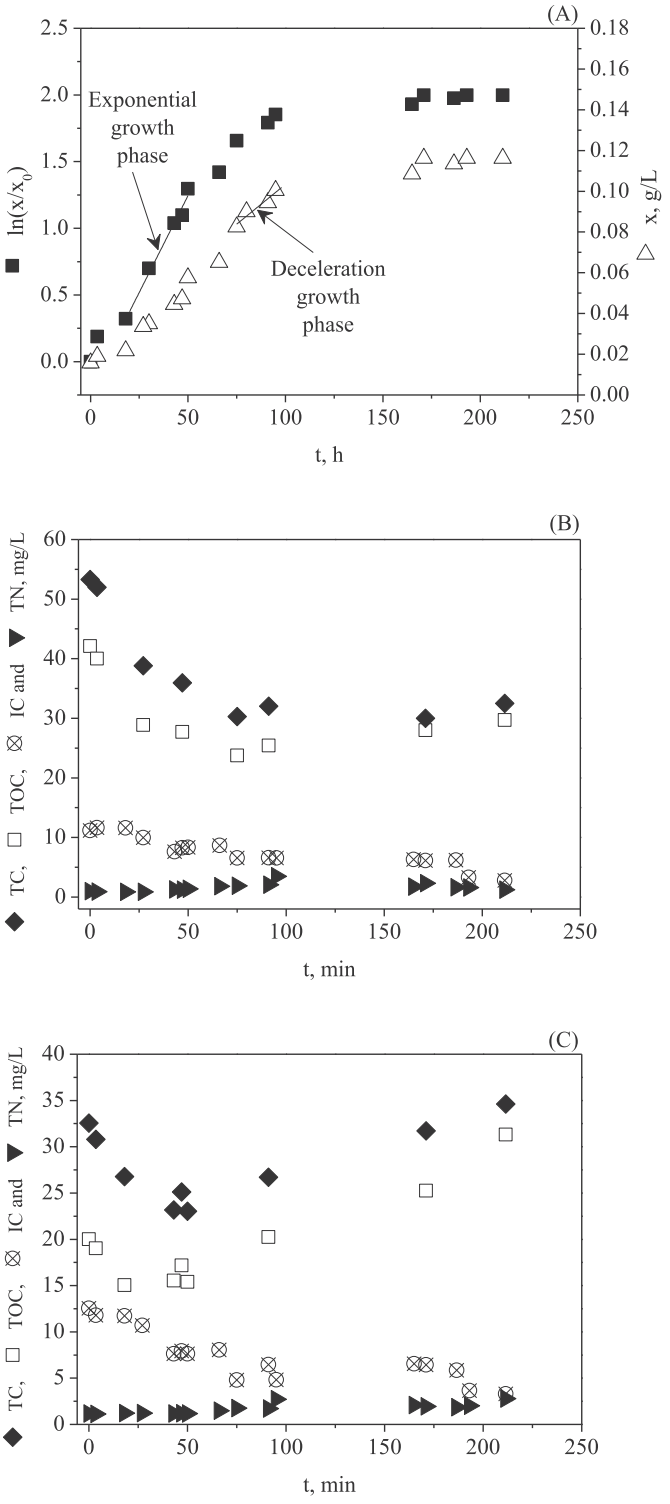

Fig. 2. *Chlorella pyrenoidosa* growth curves on 10% OMW. A) Determination of maximum specific growth rate and volumetric biomass productivity. B) Variation of total carbon species and total nitrogen on the global algal culture (algal biomass plus water treated). C) Variation of total carbon species and total nitrogen on the treated OMW (without algal biomass) during the culture.

In both cases, μ_m and P_b values were increased with the rise in % OMW in the culture medium until 50% of OMW (v/v), then these parameters were rapidly decreased (especially in the case of μ_m) indicating inhibition or toxic effect in the culture media. The

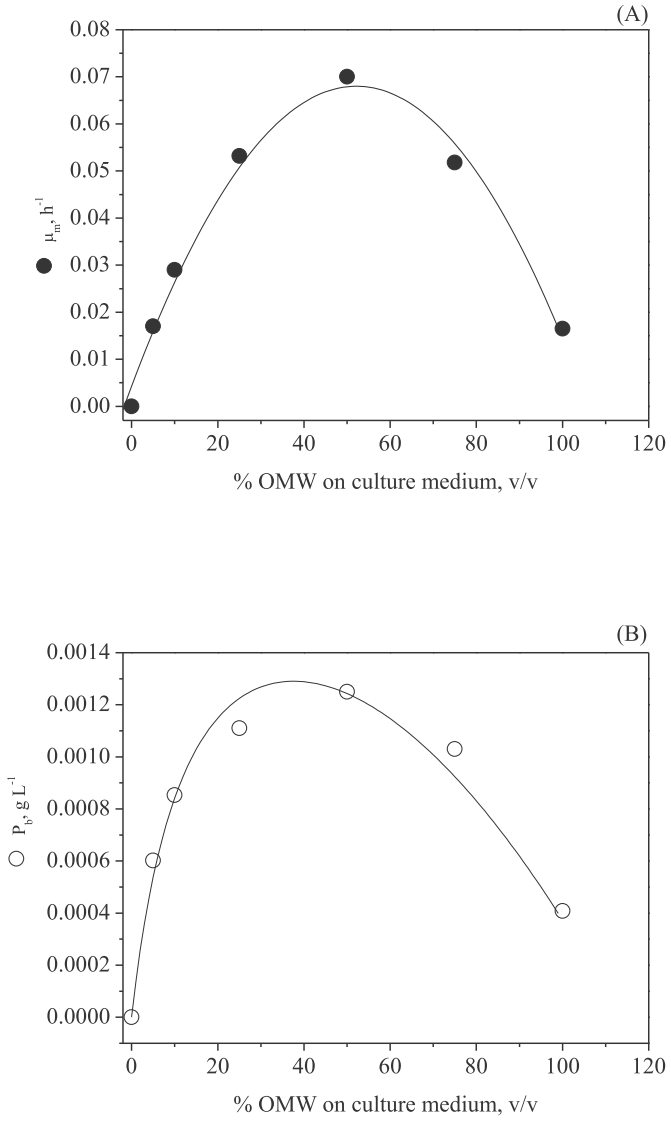

Fig. 3. Variation of maximum specific growth rates (A, red and black solid line corresponds to model type of Moser [48]) and volumetric biomass productivities (B, black solid line correspond to the modified Monod model), h^{-1}, of *Chlorella pyrenoidosa* culture in different OMW dilutions. Common operational conditions: agitation rate = 200 rpm, T = 25 °C, aeration rate = 0.5 L/min and continued illumination intensity = 359 μE m^{-2} s^{-1}.

highest experimental values of μ_m (0.07 h^{-1}) and P_b (1.25 mg/(L h)) were registered in the culture with 50% of OMW (v/v). After this concentration, these parameters were decreased to 0.0165 h^{-1} and 0.408 mg/(L h) in the culture with 100% of OMW (v/v), in which the lowest values were achieved. This result was expected due to the presence of fat matter, organics acids, pesticide residues and phenolic compounds in the composition of OMW, which are known to harm and inhibit microalgal growth (Hodaifa et al., 2012; Kobayashi and Rittmann, 1982).

After studying various inhibition and toxicity growth models by substrate, the one that best reproduced the experimental variation observed in μ_m with %OMW concentrations was the polynomial model type of Moser (1985), Eq. (3),

$$\mu_m = \mu_{m,\,max}\left(\pm\alpha_o \pm\alpha_1\,\%OMW \pm\alpha_2\,\%OMW^2\right) \quad (3)$$

where '$\mu_{m,\,max} = 0.068\ h^{-1}$' is the maximum value of the maximum specific growth rate obtained in the different cultures performed and the constant values of 'α_o, α_1 and α_2' are equal to 0.0588, 0.0367 and -3.52×10^{-4}, respectively. The parameters of the goodness of the fit were $r^2 = 0.978$ and residual sum squares (RSS) = 5.51×10^{-5}. In this sense, it is interesting to indicate that the maximum value for μ_m obtained by the mathematical model is similar to that achieved experimentally (0.07 h^{-1}).

The volumetric biomass productivity was determined by the fit of the x-t data during the deceleration growth phase, as mentioned before. The start of this phase is associated with limited availability of CO_2 (Goldman et al., 1981), light (Evers, 1990) or both, and these two components of the culture were provided at a constant rate. CO_2 was supplied through aeration of the culture medium at 0.5 v/v/min and the incident intensity of illumination was also constant in all experiments and equal to 359 μE m^{-2} s^{-1}. However, due to the colouration of the medium, the attenuation of the light was greater in culture media containing a higher percentage of OMW. This explains the decrease in P_b with the increase of OMW concentration in the culture medium. Just as with μ_m, P_b increases with the rise in OMW in the culture medium until 50% of OMW (v/v), when the maximum biomass productivity, equal to 1.25 mg/(L h), was achieved.

The model that justifies the variation of P_b with the percentage of OMW is the modified Monod model in which the presence of toxic agents or a substance at high enough concentrations were considered (Fig. 3B). This model includes a term of 'K_i %OMW^2' to describe the inhibitory or toxic effect of a nutrient at high concentrations and it is defined by Eq. (4)

$$P_b = \frac{P_{b,\,max}\ \%OMW}{K_S + \%OMW} - K_i\,\%OMW^2 \quad (4)$$

where '$P_{b,\,max} = 0.002041$ g/(L h)' is the apparent maximum value of volumetric biomass productivity without inhibition effect. Though the value of $P_{b\ max}$ is higher, the constant values of $K_S = 13.8\%$ and $K_i = 1.42 \times 10^{-7}\%$ are consistent with the data obtained experimentally. The parameters of the goodness of the fit were $r^2 = 0.961$ and residual sum squares (RSS) = 3.22×10^{-8}. P_b values were similar to that registered by Sánchez et al. (2001). In that work, *C. pyrenoidosa* was cultivated in OMW obtained from a continuous olive oil extraction system using 'Decanter' with three exits, this OMW is known as 3-phase system or 'Alpechín' in Spain. However, lower μ_m values (0.011–0.045 h^{-1}) were obtained due to the higher organic matter concentration in OMW from three-phase extraction system (DQO = 40–220 g O_2/L) in comparison with OMW from two-phase extraction system (DQO = 4–16 g O_2/L), (Agabo-García and Hodaifa, 2017).

3.3.2. Biochemical composition of C. pyrenoidosa biomass

At the end of the experiments, the harvested biomass of *C.pyrenoidosa* was analysed for proteins, carbohydrates and lipids contents determination. These are the microalgae cells main components. The variation on the biomass composition of *C.pyrenoidosa* for all OMW dilutions is shown in Fig. 4.

Microalgal cells require nitrogen for the synthesis of protein, nucleic acid and phospholipids, and thus the growth of microalgae is believed to be essential for nitrogen removal (Wang et al., 2015). Protein content of the microalgae biomass was increased with the increment of OMW concentration in the culture media (Fig. 4) and ranged from 0.99% (Initial $TN_{culture\ medium} = 0.948$ mg/L and $\%TN_{final\ biomass} = 0.155\%$), in 5% OMW (v/v) culture media, to 51.5% (Initial $TN_{culture\ medium} = 17.3$ mg/L and $TN_{final\ biomass} = 8.25\%$), in 100% OMW culture medium. It could therefore be concluded that protein content of the microalgae cells was sensitive to changes in nutrient levels. The initial nitrogen content in the low concentration OMW

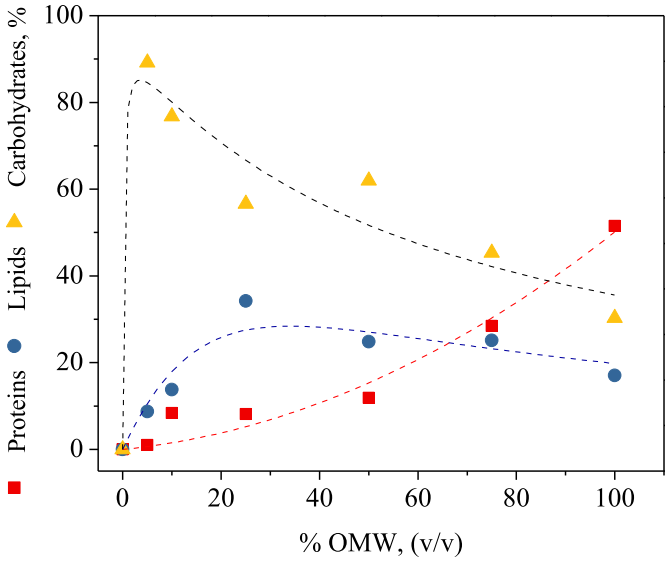

Fig. 4. Variation of biochemical composition of *C. pyrenoidosa* biomass with the augment of real OMW percentages on the culture media.

culture medium was not enough for the synthesis of proteins, causing the decrease of the protein content in the biomass at the end of the culture and in the microalgae growth subsequently. Proteins are essential for microalgae growth. Nutrient deficiency could inhibit protein synthesis and microalgae growth subsequently. Zhang et al. (2017) demonstrated the rapid biomass accumulation of *C. pyrenoidosa* when the microalgae was grown in straw hydrolysate medium and the effectiveness of nitrogen regulation in biomass composition in heterotrophic condition. Hodaifa et al. (2008) obtained similar results with the same OMW and *Scenedesmus obliquus*. In this study, the percentage of protein varied between 6.2% and 30.8%, corresponding to 5% and 50% OMW (v/v) culture media. The biomass protein content of *S. obliquus* reached a value of up to 43.8% (Hodaifa et al., 2013) when the microalgae was cultured in a medium without N deficiency as the Rodríguez-López (1964) synthetic medium (Becker, 1994).

Carbohydrates content in biomass under low OMW percentages increased because of nutrient deficiency (mainly nitrogen). Under nitrogen stress condition, microalgae store carbohydrates as molecular reserves that can be used as alternative energy sources. This is consistent with previous findings showing that carbohydrate accumulation in microalgae is triggered by nitrogen depletion. On the other hand, cultures with 5% OMW are virtually transparent after primary treatment, which favoured autotrophic culture. In this sense, through photosynthesis microalgae can convert atmospheric CO_2 along with water and light into organic matter, being carbohydrates the major products. The excess of fixed carbon is commonly stored into carbohydrates, and in stressful conditions, these molecular reserves can be used as alternative energy sources for the production of cell structures (Wang et al., 2015).

In terms of lipids content in *C. pyrenoidosa* biomass, it ranged from 8.71% (5% OMW, v/v) to 34.21% (25% OMW, v/v). In all experiments carried out, the total nitrogen in OMW after primary treatment were varied from 0.489 mg/L (5% OMW, v/v) to 17.3 mg/L (100% OMW, v/v). Nevertheless, the initial TN availability in control synthetic medium of Rodríguez López was = 140 mg/L (Ródriguez-López, 1964). This fact indicated that all experiments in this work were performed under nitrogen stress condition. On the other hand, these results are consistent with those obtained in previous studies in which microalgae were cultivated under stress conditions such as high OMW concentration, nitrogen and phosphate limitation or high salinity. In stress conditions, lipids formation are preferred storage compounds due to its high-reduced state and were packed in cells for the microalgae survival (He et al., 2015; Wang et al., 2015; Yao et al., 2015).

Table 2 shows the identified fatty acids in the algal biomass lipid fraction harvested from the different culture media. Fatty acids were grouped into saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA). In general, higher SFA percentages (85.2%–95.1%) were registered. Moreover, a slightly increase in the SFA percentages was appreciated with the augment of %OMW (more darkness caused by colour effect) in the culture media. The attenuation of light by the gradual change in cultures colour was greater with higher %OMW. Fact that allowed the change of culture behaviour from mixotrophic to heterotrophic. In this sense, Hodaifa et al. (2009) observed for *S. obliquus* biomass that SFA content was higher in the absence of light (heterotrophic growth) than in the presence of light (mixotrophic light-limited cultures). Mixotrophic with high light inhibition and heterotrophic cultures behaved similarly, and the content of SFA approached and even exceeded the heterotrophic value, regardless of the aeration supplied. On the other hand, MUFA and PUFA contents showed the opposite trend, as contents were greater in mixotrophic (low %OMW) than in heterotrophic (high %OMW) cultures (Hodaifa et al., 2009). It is necessary to indicate that the higher percentage of SFA in 5% OMW (v/v) culture is due to the few fatty acids identified in the lipid fraction of the biomass. This fact could be explained considering the small amount of algal biomass obtained (0.980 mg/L) at the end of the culture.

The main fatty acids found were palmitic acid (16:0), oleic acid (18:1n9) and stearic acid (18:0). Palmitic acid has been registered the highest percentages (65.7%–74.7%). On the contrary, palmitoleic acid (16:1) was only detected in experiments with 50% (v/v) of OMW. The only polyunsaturated acid identified was 18:2n6 and it was detected in the biomass obtained from all experiments. Higher percentages of linoleic acid (18:2n6) were found in the biomass obtained from low OMW concentration cultures (5% OMW, v/v), but no linolenic (18:3n3), EPA (20:5n3) or DHA were found in any of the experiments. Obtaining a high lipid fraction (34.2% in the culture with 25% OMW, v/v) in the final biomass gives rise to the possibility of using this fraction for biodiesel production. In this sense, special attention must be paid to the linolenic acid (18:3) and other polyunsaturated fatty acids (≥4 double bonds) content of the

Table 2
Fatty acid profiles obtained from the lipid fraction of *C. pyrenoidosa* biomass at the end of the experiments.

Fatty acids	Olive-oil mill wastewater concentration, % (v/v)					
	5	10	25	50	75	100
C14:0	n.d	0.80	0.61	0.48	0.56	1.56
C16:1	n.d	n.d	n.d	0.19	n.d	n.d
C16:0	71.9	66.5	74.7	65.7	72.3	63.7
C18:2*n*6	5.43	0.29	0.19	0.31	0.45	0.24
C18:1*n*9	4.66	13.9	4.70	14.3	7.32	8.32
C18:0	10.8	7.11	7.75	8.11	8.60	7.31
C20:0	n.d	2.69	2.91	2.46	1.67	2.49
C22:0	n.d	0.84	0.66	0.40	0.55	1.56
C24:0	n.d	0.62	0.66	0.60	0.67	2.30
C26:0	n.d	4.57	5.20	4.24	5.09	8.36
C28:0	n.d	2.68	2.65	3.24	2.83	4.20
ΣSFA[a]	89.9	85.8	95.1	85.2	92.2	91.4
ΣMUFA[b]	4.66	13.9	4.71	14.5	7.32	8.32
ΣPUFA[c]	5.43	0.29	0.19	0.31	0.45	0.24

[a] Corresponding to the sum of saturated fatty acids.
[b] Corresponding to the sum of monounsaturated fatty acids.
[c] Corresponding to the sum of poly unsaturated fatty acids.

biomass since the European Standard (EC, 2008) specifies maximum limits of 12.0% and 1%, respectively, for a good biodiesel quality production. All lipid fractions obtained in the experiments are close to that specified by the European Standard (EC, 2008). It is necessary to indicate that higher percentages of saturated fatty acids in the lipid fraction give more stability to the produced biodiesel since these fatty acids are not prone to oxidation.

In any case, the final biomass obtained (0.098143 mg/L-0.143 mg/L) could be used in combination with other substrates for biofuels production or maybe as supplementary substrate in the anaerobic digester for biogas production. In addition, as a last option, it could be used for domestic, commercial or industrial boilers and as a fuel for generators to produce electricity.

3.3.3. OMW degradation by microalgae and final treated water quality

Microalgae have the ability to consume organic and inorganic nutrients for cell generation. In this work, the biological treatment proposed was based on *C. pyrenoidosa* growth. Fig. 2B shows total carbon species and total nitrogen variation in the global algal culture (OMW + microalgal biomass). A decline in the total organic carbon during the first stages of the culture, corresponding with the exponential growth of *C. pyrenoidosa,* is due to the organic compounds removal from the culture medium and its conversion into biomass structures. Once the exponential and linear growth phases were finished, the concentration of TC and TOC showed a slight rise explained by the assimilation of smaller quantities of organic compounds due to the cessation of growth and the microalgae death and cell ruptures.

Fig. 2C shows the variation of all carbon species concentration with time in the treated OMW (culture medium) without microalgal biomass. It can be observed a rapid decrease in the starting period, particularly in the first 50 h, corresponding this descent with the exponential growth phase of the microalgae. This result pointed out that total organic matter removal efficiency was dramatically increased during the exponential phase and indicated that the microalgae was able to assimilate organic compounds as a carbon source through mixotrophic metabolism when both organic carbon and light are present. An increase of the TOC and TC at later stages of cultivation is associated with cell death and ruptures, which leads to an increase in the content of organic compounds in the medium.

In all experiments, IC concentrations (in treated OMW and global culture) were decreased with time (Fig. 2B and C). The reduction of the IC levels during the first 50 h of the culture in parallel with TC and TOC concentrations can be explained by the ability of *C. pyrenoidosa* to grow mixotrophically assimilating organic compounds as carbon sources while using inorganic compounds as electron donors (Chojnacka and Marquez-Rocha, 2004).

After exponential growth, when all the assimilated organic compounds (mainly sugars) were removed, the reduction of IC levels during the last hours of the culture (treated OMW, Fig. 2C) can be explained by the assimilation of inorganic carbon and light by microalgae.

Table 1 shows the treated water characteristics after microalgae growth. In general, for all experiments and characterization parameters, higher removal percentages were registered in cultures in which larger OMW dilutions were used. In this sense, the removal values of %TC, %TOC, %IC and %TN were 74.0%, 75.5%, 71.3% and 87.6%, respectively, in the culture medium formed by 25% of OMW (v/v). These values were decreased to 23.3%, −15.5%, 63.1% and 67.3%, respectively, in the experiment in which undiluted OMW was used. This COD and TOC reduction was observed in the six different culture media, indicating that the microalga was able to use organic carbon and light throughout mixotrophic metabolism. All parameters were decreased throughout the secondary OMW treatment process, with the exception of turbidity and TOC in the culture without OMW dilution, which showed an increase after *C. pyrenoidosa* culture due to the presence of cell debris in the final treated water. In this sense, it is interesting to indicate that after carrying out multiple centrifugations of the supernatant obtained after the first separation by centrifugation of the cell suspension of microalgae, a drop of approximately 30% in the parameters of COD and TOC (data not shown) was observed. The behaviour of removal percentages registered for characterization parameters is consistent with the variation of the maximum specific growth rates and biomass productivities values (Fig. 3).

To determine the effectiveness of the secondary treatment for phenols degradation, their content in the OMW was determined after algal growth. In general, TPCs were decreased through the course of the culture. Furthermore, a steeper decrease can be observed during the exponential phase of growth (Fig. 5A). TPCs removal percentages increased with the augment of %OMW (v/v) in the culture medium. These values were increased from 58.6% to 67.1% in the cultures with 5% and 50% OMW (v/v), respectively, and showed a decrease to 36.4% in the culture constituted by undiluted OMW (Fig. 5B). This behaviour is consistent with the observed variation of the μ_m and P_b values with %OMW in the culture media. In addition, it is interesting to indicate that *C. pyrenoidosa* biomass was able to degrade the majority of the TCPs (final TPCs < 1 mg/L) present in the culture medium when the initial concentration was below 5.4 mg/L. The highest algal concentration was achieved when initial TPCs content in the culture medium was lower or equal to this value.

Fig. 5B shows the variation of the final %TPCs removal registered in the different culture media. In this sense, many authors have demonstrated the ability of *C. pyrenoidosa* to eliminate high concentrations of phenols and other polluting compounds. Dayana and Bakthavatsalam (2016, 2017) investigated the degradation effect of *C. pyrenoidosa* (KX686118) on the phenolic effluent of a coal gasification plant. In these previous works, final concentrations of phenols of up to 1.1 g/L were achieved after microalgae growth, registering removal percentages higher than 90%. In addition, Wang et al. (2015) studied triclosan removal and biodegradation in water by using the same microalgae. When *C. pyrenoidosa* was exposed to a series of triclosan concentrations ranging from 100 to 800 ng/mL, more than 50% of triclosan was eliminated by algal uptake from the culture medium during the first 1 h of exposure, reaching the equilibrium after 6 h treatment. In biodegradation experiments, a removal percentage of 77.2% was obtained after the *C. pyrenoidosa* culture in the presence of 800 ng/mL triclosan for 96 h. In addition, Lika and Papadakis (2009) demonstrated that biodegradation of phenolic compounds by microalgae occurs in a shorter time interval during the first stages of cultivation, when all nutrients required by the microalgae are present in the culture medium. When algal cells are grown under constant light intensity and in the presence of organic compounds as carbon source (mainly carbohydrates), there is a substantial increase in the growth resulting in higher biomass, this exponential growth phase corresponds with the stage when the bioremoval of the phenolic compounds by the microalgae is performed. In this context, it is important to indicate that carbohydrates and phenolic compounds uptake is performed by microalgae. In this sense, Di Caprio et al. (2018) when studying biodegradation of OMW sugars by the green microalga *Scenedesmus* sp. indicated that phenol removal took place immediately after the stop in the consumption of OMW sugars.

At the end of the process, a high quality treated water was obtained and did not present any toxicity considering that it comes from a combined process where ultraviolet light is applied (which has a disinfecting effect) and microalgae are grown. Parameters

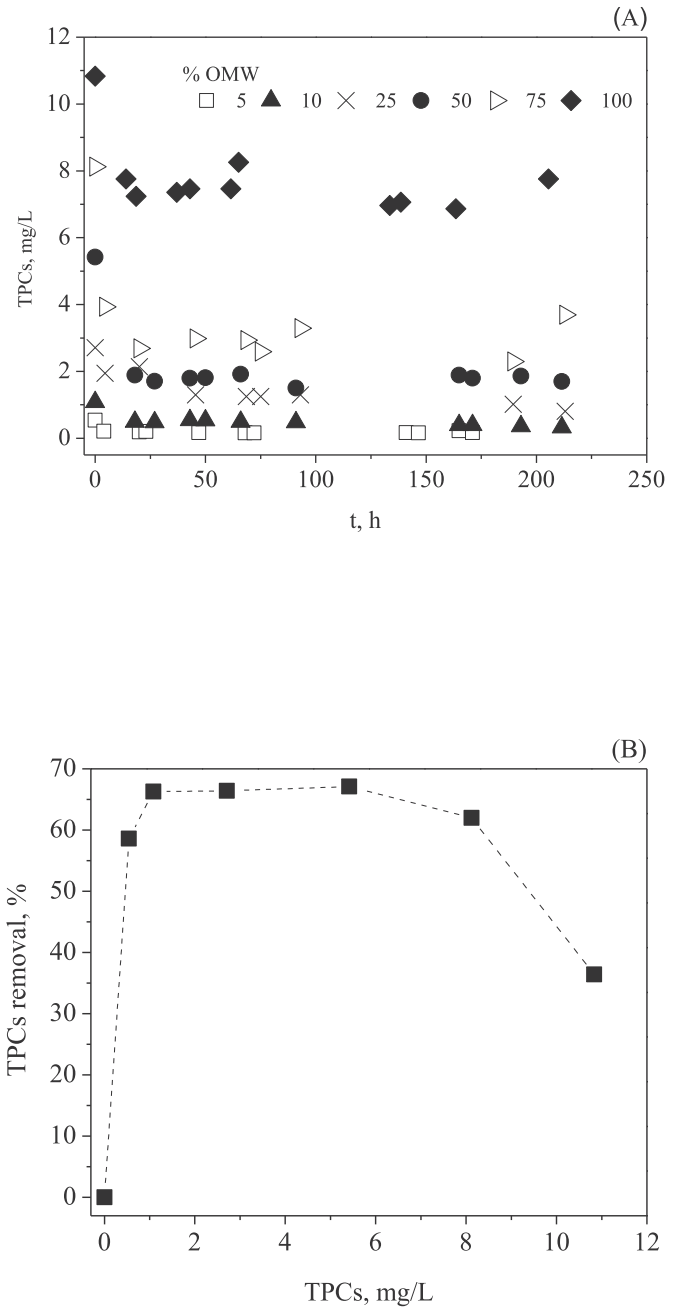

Fig. 5. Variation of total phenolic compounds concentration (A) and final removal percentages of TPCs (B) versus time and TPCs initial concentrations, respectively.

registered in Table 1 indicate that treated water could be used for irrigation and discharges to surface water and groundwater or for drinking water.

Spanish environmental standards for treated OMW intended to be used as irrigation water, established that treated water must comply the following parameters pH = 6–9, suspended solids <500 mg/kg and COD <1000 mg O_2/L (Resolution of Guadalquivir River Basin president, 2006). In addition, the treated water at the exit of the process comply with European Directive 91/271/EEC where COD <125 mg O_2/L and TN = 10 mg/L for treated water discharge into receiving waters (European Commission Directive, 1991).

On the other hand, the consolidated text of the Drinking Water Directive with its latest amendments, including Commission Directive (EU) 2015/1787 of 6 October 2015, define that drinking water is all water used in any food-production process undertaking for the manufacture, processing, preservation or marketing of products or substances intended for human consumption unless the competent national authorities are satisfied that the quality of the water cannot affect the wholesomeness of the foodstuff in its finished form. This Directive established chemical parameters and indicator parameters which determined the drinking water quality. These are electric conductivity <2500 μS/cm, turbidity acceptable to consumers and no abnormal change, TOC = no abnormal change, iron = 0.2 mg/L, sulphate = 250 mg/L, sodium = 200 mg/L and ammonium = 0.5 mg/L. The values obtained for treated OMW from crude OMW concentration <25% (v/v) have values next to that request by drinking water standards. In any case, if some parameter needs to be adjusted some other units such as ion exchange unit or other membrane technology units could be added.

4. Conclusion

OMW have a complex composition, which hampers its treatment. The combined process based on physico-chemical and biological treatments is essential for its efficient treatment. The primary treatment (flocculation, photolysis and microfiltration) allowed the elimination of a large part of OMW organic load (96.2% of COD, 80.3% of TOC and 96.6% of TPCs). Secondary treatment eliminated the rest of OMW organic load and the final treated water is suitable to be used for irrigation, discharge to receiving waters or for its reuse in the process itself allowing the closing of water cycle in the factory. The low percentage of sludge generation (mainly during flocculation) can be recirculated to the head of the treatment process or be directly used in composting. After the primary treatment, higher growth rates for *C. pyrenoidosa* ($\mu_m = 0.07$ h^{-1} and $P_b = 1.25$ mg/(L h)) were registered. Final biomass obtained may be used in direct combustion, methane production or in biodiesel production.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Combination of physicochemical operations and algal culture as a new bioprocess for olive mill wastewater treatment

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ABSTRACT

This work presents a new bioprocess design which allows a substantial reduction of organic and inhibitory compounds and a better quality of the final treated water. The process involves a physicochemical (primary) and a biological (microalgae) treatment, which were tested separately with lab equipment, for olive oil mill wastewater (OMW). Primary treatment of OMW involved flocculation-sedimentation by Flocudex CS-51 and microfiltration using a 0.2 μm membrane. Secondary treatment consisted of *Scenedesmus obliquus* culture in different OMW dilutions in ultrapure water as culture media: 5, 10, 25, 50, 75 and 100%. Experiments were performed on a laboratory scale in stirred batch tank reactors. The common operating conditions were: pH = 7, temperature = 25 °C, agitation rate = 3.33 Hz, aeration rate = 0.5 min^{-1} and illumination intensity = 359 μE m^{-2} s^{-1}. High global removal levels were achieved after primary treatment for chemical oxygen demand (92.6%), total phenolic compounds (98.9%), total organic carbon (75.9%), total nitrogen (63.5%) and inorganic carbon (55.3%). Similar results were obtained for the main OMW constituents after secondary treatment with final harvested biomass rich in energetic compounds, where the highest values of carbohydrates (72.5%) in culture with 5% OMW and lipids (44.9%) in 100% OMW culture were determined.

1. Introduction

Microalgae can be considered as the microorganisms of the future due to their potential in numerous applications. By way of example, they are sustainable bioremediation agents and a source of energy, proteins, natural pigments, etc. In addition to its use in cosmetics, pharmaceutical applications, human and animal feed, aquaculture, etc. [1].

Microalgae are promising microorganisms characterized by its easy culture, high growth rate and biomass productivity. In addition, microalgae can grow in simple conditions with solar light and inorganic nutrients. The use of synthetic media for microalgae cultivation at industrial scale is economically unviable due to the high costs of chemicals. This fact implies the need to seek cheaper alternatives to form culture media. In this sense, the use of waste and its transformation into by-products for the microalgae cultivation is a good alternative [1,2].

Generally, wastewaters have macro, micro and trace nutrients that can be used by microalgae. Double goals can be achieved: wastewater treatment and generation of biomass with high economic value. In brief, it is a sustainable and eco-friendly bioprocess [2]. Species such as *Ankistrodesmus falcatus*, *Botryococcus terriblis*, *Chlorella pyrenoidosa*, *Scenedesmus obliquus* or *Spirulina platensis* have shown an efficient growth and high removal rates of contaminants (heavy metals, pesticides, etc.) contained in many wastewaters as urban and those generated by industries such as aquaculture, soybean processing, dairy industries, etc. [3].

Industrial wastewaters are heterogeneous and complex since they contain suspended solids, chemicals, greases, etc., which can lead to growth inhibition. In this sense, the correct design of the bioprocess is key to achieve the highest removal of organic and inorganic load from wastewater. At the same time, a proper bioprocess design allows a more rapid microorganism growth and higher biomass production [4,5].

In conventional wastewater treatment, different stages are generally applied. Primary treatment is intended to eliminate large solids and particles. Secondary seeks to the bioremediation of organic compounds through the action of microorganisms. In addition, in some countries, a tertiary treatment is applied to reuse the final treated water [5]. Olive mill wastewaters (OMW) are one of the most polluting within the agro-food industry waste, constituting a major concern in the

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Mediterranean area, where 30 hm^3 of OMW are generated per year. Press, batch, and continuous methods are used for olive oil extraction. Nowadays, continuous methods (two and three-phases) are used in most of the producing countries. In both cases different wastewater biochemical composition is obtained [6,7]. In general, OMW has a dark brown colour, unpleasant odour, low pH, high turbidity, organic load, polysaccharides, sugars, proteins and phenolic compounds such as hydroxytyrosol, tyrosol, *p*-hydroxyphenyl acetic acid, *p*-coumaric acid and caffeic acid, etc. [8–10]. Phenolic compounds (e.g. > 5 mg/L become toxic for *Chlorella pyrenoidosa*) are responsible for the phytotoxic effect and antibacterial activity of OMW, which causes eutrophication, pollution of soils and water resources [11]. Currently, OMW storage in evaporation ponds is the most common practice for its management. This system, based on the water removal by evaporation, does not provide a solution for the remaining solid phase. Additionally, it leads to the contamination of water resources and the generation of bad odours [7]. Another alternative proposed and used in some countries is the direct spread on agricultural lands. However, not all countries have this option in its legislation due to the great impact of OMW on soils properties such as pH, electric conductivity, nitrogen and phosphorous availability, etc. [12,13]. For this reason, several researchers have proposed physicochemical (sedimentation, flocculation, etc.) [14], biological (aerobic activated sludge [15], anaerobic digestion [16], composting [17], etc.), membrane filtration (micro-, ultra- and nanofiltration) [18] and chemical oxidation methods (Fenton [19], Photo-Fenton [8], ozonisation [20], TiO_2 photocatalysis [21], etc.). In this sense, Paraskeva et al. [18] combined natural sedimentation, ultrafiltration, nanofiltration and reverse osmosis and recuperated the solid fraction, the phytotoxic fraction with high molecular weight, water for fertilization (nutrient fraction) and a second concentrated phytotoxic fraction with the potential to be used as growth inhibitors of some native plants, respectively. Markou et al. [22] obtained a microalgae biomass (*Spirulina platensis*) rich in carbohydrates and proteins after OMW pretreatment with sodium hypochlorite. Malvis et al. [11] combined flocculation, photolysis and microfiltration with algal culture (*Chlorella pyrenoidosa*) for OMW treatment and generation of microalgae biomass rich in energetic compounds.

This research aims to study the ability of *Scenedesmus obliquus* to use two-phases OMW as a substrate by reusing its nutrients. In this sense, two goals are achieved: OMW bioremediation and valuable biomass generation. Primary and secondary treatments are designed to accomplish these purposes. Primary consists of flocculation-sedimentation unit to eliminate solids, turbidity and part of OMW colour, followed by microfiltration unit with 0.2 μm membrane to remove organic colloidal matter. Secondary treatment consists of microalgal cultures (5, 10, 25, 50, 75 and 100% of OMW/water). Then, kinetic parameters such as specific growth rates and volumetric biomass productivities were determined. Final biomass value was evaluated through the biochemical composition. Furthermore, the treated water quality during and at the end of the process was determined.

2. Materials and methods

2.1. Microorganism and photobioreactor

The microorganism used in this work was the freshwater green microalga *Scenedesmus obliquus* CCAP 276/3A. Stock cultures were maintained in solid Rodríguez-López Medium [23] solidified with agar. Then, cultures were maintained at room temperature and continuous artificial illumination.

Experiments were performed in sterile conditions, at laboratory scale, in stirred batch tank reactors with 1 L work volume and 10 cm (diameter) × 16 cm (high) dimensions. All material and glass bioreactors were sterilized in an autoclave at 121 ± 1 °C for 30 min. Culture media were sterilized by membrane filtration using a membrane of cellulose nitrate with 0.2 μm (pore size).

2.2. Culture media

OMW was taken from a reservoir of an olive oil mill with continuous centrifugation process using a decanter with two outlets (olive oil and pomace). The olive oil extraction plant was in Seville (Spain). The flocculation-sedimentation was performed during 90 min in a 1 L Imhoff cone using a commercial flocculant (Flocudex CS-51). Optimal flocculant has been chosen at 100 mg/L according to a previous study of Hodaifa et al. [14]. The mixture of flocculant with OMW was carried out in two stages. First, high stirring rate at 11.7 Hz was applied for 1 min to perform fast and uniform mixing of flocculant with the OMW. Second, slow stirring rate at 5.83 Hz during 30 min was performed to allow the formation of flocs and increase their size.

Flocculated OMW (F-OMW) was used to form the culture media (F-OMW/Ultrapure water) at different concentrations 5, 10, 25, 50, 75 and 100%. Microfiltration through a 0.2 μm membrane was used for the removal of colloidal particles and culture media sterilization. The pH of culture media was adjusted to an initial value of 7.0 with 0.1 mol/dm^3 NaOH and 0.1 mol/dm^3 HCl solutions.

The common culture conditions used were: temperature = 25 °C, aeration rate = 0.5 min^{-1}, pH value = 7.0, agitation rate = 3.33 Hz and artificial continuous white light with illumination intensity = 359 μE m^{-2} s^{-1}. A cell suspension from sterile Rodríguez-López Medium [23] was used as initial inoculum for OMW cultures at 0.00405 ± 0.00236 g/L.

2.3. Physicochemical characterisation of raw industrial olive mill wastewater

The high complex composition of OMW hampers its treatment [24]. The main physicochemical characteristics of raw OMW used in this work are summarized in Table 1. The parameters turbidity = 714 FTU, chemical oxygen demand (COD) = 5839 mg/L, total phenolic compounds (TPCs) = 322 mg/L, total organic carbon (TOC) = 328 mg/L and total nitrogen (TN) = 58.9 mg/L, represent the organic matter, the main parameter to consider from the environmental point of view. High concentration of carbon and nitrogen is desirable since both are required nutrients for microalgae growth. Raw OMW presents approximately half the concentration (2.4 times) of total nitrogen than the mineral synthetic medium of Rodríguez-López [23], with 140 mg/L, which is normally used as control medium for the same microalgae [25]. TPCs were transferred to the industrial raw OMW during olives crushing and olive oil washing [8].

In addition, OMW also contains inorganic salts measured as inorganic carbon (IC) = 318 mg/L and orthophosphate (PO_4^{-3}) = 43.1 mg/L. Phosphorous concentration in raw OMW is notably lower than that of Rodríguez-López, with phosphorous = 160 mg/L [26]. The presence of orthophosphate is highly relevant in metabolism phosphorylation reactions [27].

Chloride has been shown to be toxic for microalgae growth at high concentrations. In this sense, Figler et al. [28] proved for *S. obliquus* cultured in Bold's Basal medium, that 5.8 g/L of NaCl (3.51 g/L of Cl^-) caused 50% growth inhibition (EC_{50}) after 4 days, and concentrations higher than 10 g/L of NaCl (6.1 g/L of Cl^-) were toxic. In addition, according to Li et al. [29], this value for *Chlorella pyrenoidosa* ranged from 19.7 g/L to 36.3 g/L. The chlorides concentration in raw OMW used in this work is only 204 mg/L and 98.5 mg/L, after primary treatment, at the beginning of *S. obliquus* cultures, so the growth of *S. obliquus* is adapted/inhibited at this low concentration.

In addition, sulphur, a required component of some amino acids, vitamins and sulfolipids, was detected at high concentration in the form of sulphate (320 mg/L). Iron (1.19 mg/L) is necessary for photosynthesis, due to its role in enzymatic reactions in photosystem I and II. Furthermore, it is a key factor in the synthesis of essential proteins such as ferredoxin and cytochrome [27,30]. Several studies have shown the effect of iron concentration on the biomass and lipid content in different

Table 1
Characterisation of wastewater used before and after treatment by flocculation and microfiltration.

Parameter	Raw OMW	Primary treatment		Secondary treatment
		Flocculated	Microfiltration	S. obliquus
pH	6.25 ± 0.8*	Natural**	Natural	8.9 ± 0.1
Conductivity, mS/cm	1.97 ± 0.5	1.30 ± 0.2	1.44 ± 0.2	6.8 ± 0.1
Turbidity, FTU	714 ± 6.0	53.5 ± 2.1	4.09 ± 1	25.6 ± 0.6
COD, mg/L	5839 ± 60	2484 ± 11	433 ± 10	192 ± 5
TPCs, mg/L	322 ± 3.0	4.2 ± 0.1	3.62 ± 0.2	2.33 ± 0.2
TC, mg/L	646 ± 27	561 ± 11	222 ± 7	148 ± 6
TOC, mg/L	328 ± 2.0	530 ± 8.0	79.2 ± 6	62.9 ± 7
TN, mg/L	58.9 ± 3.6	27.8 ± 0.7	21.5 ± 1	5.99 ± 0.6
IC, mg/L	318 ± 4.0	31.3 ± 1.3	142.3 ± 2	85.1 ± 0.4
Iron, mg/L	1.19 ± 0.01	1.10 ± 0.1	0.67 ± 0.01	0.72 ± 0.02
Chloride, mg/L	204 ± 4.0	116 ± 4	98.5 ± 1.3	156 ± 6
Sulphate, mg/L	320 ± 30	84.8 ± 2.9	53.8 ± 1.1	56.8 ± 0.3
Sodium, mg/L	0.943 ± 0.1	0.782 ± 0.02	0.05 ± 0.005	0.99 ± 0.12
Orthophosphate, mg/L	43.1 ± 2.1	21.7 ± 1.3	21.3 ± 2	9.24 ± 0.46

*Standard deviation value.
**pH without modification.

microalgal species. Liu et al. [31] proved that increasing the iron concentration in the medium caused an increase in the content of biomass and lipids in *Chlorella vulgaris*. Additionally, Abd El Baky et al. [32], got a lipid content increase in *Scenedesmus obliquus* from 5.6% to 28% by increasing the iron concentration in the culture medium.

2.4. Analytical methods

The following parameters were determined for raw and treated OMW: pH value, electric conductivity (EC), turbidity, chemical oxygen demand (COD), total phenolic compounds (TPCs), total carbon (TC), total organic carbon (TOC), total nitrogen (TN), inorganic carbon (IC), total iron, chloride, sulphate, sodium and orthophosphate.

pH, electric conductivity (EC) and turbidity values were directly measured by using a pH-meter Crison, mod. GLP 22C, Conductimeter Crison, mod. GLP31 and Turbidimeter Hanna, mod. HI93703, respectively.

Chemical oxygen demand was determined photometrically at 620 nm according to German standard methods [33].

The determination of total phenolic compounds was performed by making it react with a derivative thiazol, giving a purple azo dye, determined photometrically at 475 nm according to the standard methods [34,35].

Total carbon, total organic carbon, inorganic carbon and total nitrogen concentrations were determined using a Total Carbon and Nitrogen Analyser provided by Skalar Company, mod. Formacs^{HT} and Formacs^{TN}.

Total iron ions determination was performed through the reduction of all iron ions to iron (II) ions in a thioglycolate medium with a derivative of triazine. This reaction results in a reddish-purple complex that was photometrically determined at 565 nm according to the standard methods [34,35].

Sulphates and orthophosphates were determined photometrically at 420 nm and 690 nm, respectively, according to the standard methods [34,35].

Sodium content was directly determined by using a selective ion electrode for each ion (Crison, mod. GLP 22C).

Furthermore, biomass generated and biomass biochemical composition were determined. For biomass concentration (x, g/L), a volume of 5 ml of microalga suspension was taken and centrifuged (Relative centrifugal force = 4226) at 50 Hz for 10 min. The obtained biomass pellet was washed three times with ultrapure water and measured at 600 nm in a UV–visible Spectrophotometer. A linear calibration curve between absorbance and dry biomass was established. In this sense, a linear relationship from the experimental data of dry weight-cell concentration (g/L) versus absorbance was obtained. The experimental data were determined from samples taken during and at the end of all *S. obliquus* cultures.

Total pigments (chlorophyll *a*, chlorophyll *b* and carotenoids) were determined by a photocolourimetric method after its extraction with 90% acetone as described by Ritchie [36]. The total chlorophylls and total carotenoids contents were calculated according to the equations described by Jeffrey and Humphrey [37] and by Strickland and Parsons [38], respectively.

At the end of each culture, biomass was separated and dried at 105 °C. Then, total lipids, proteins and fatty acids content were determined.

The total lipid content of the biomass was extracted by a micro-soxhlet extractor using n-hexane as solvent for 24 h.

Fatty acids (FA) identification and quantification was performed according to Lepage and Roy [39] in a gas chromatograph (Hewlett--Packard, Model 5890 Series II) equipped with a flame ionization detector through its transesterification into fatty acid methyl esters (FAME).

The crude protein content was calculated after the determination of total nitrogen concentration by a total carbon and nitrogen analyser provided by Skalar Company (mod. Formacs^{HT} and Formacs^{TN}) according to the formula provided by Becker [40], %Crude proteins = % TN × 6.25.

The total carbohydrate content was calculated by considering that proteins, carbohydrates, lipids, pigments and genetic materials (considered approximately about 1% [40]), are the main components of algal biomass.

2.5. Statistical methods applied

To confirm the reproducibility of the experimental data reported, the cultures were made at least in duplicate and the analytical methods were applied at least in triplicate. In the duplicated experiments, biomass growth was monitored, and the final wastewater quality was determined. Graphics and statistical methods used were available in OriginPro 8.0.

3. Results and discussion

3.1. Bioprocess designed for Scenedesmus obliquus growth

The complex composition of olive mill wastewater, the high organic load and the presence of compounds that inhibit the growth of microorganisms are the main factors that limit the application of conventional technologies (mainly biological treatments) on its treatment. In addition, this kind of treatments generate large quantities of sludge that must be managed, reduced, or eliminated. In fact, up to now, there is not a solution for this wastewater and it is only managed in large accumulation reservoirs for its evaporation during the summer months. Not to mention that proposed methods such as direct ozonisation, forced evaporation, etc. have a higher cost [41,42].

This work proposes the use of microalgae for olive mill wastewater treatment since it does not imply the generation of a sludge at the end of the process. In addition, the generated algal biomass has a high

economic value since it can be used for biofuels production in a substantial way, without forgetting the ability of microalgae to eliminate atmospheric carbon dioxide, contributing to the reduction of the greenhouse effect.

In order to decrease the organic matter content (precisely, COD and turbidity), including inhibitor growth compounds (phenolic compounds) in the wastewater, it is necessary its treatment before being used in algal cultures. In this sense, Floculdex CS-51, a cationic polyelectrolyte (organic polymer for food use) with high molecular weight, soluble in water and based in polyacrylamide, was used based on its great capacity to remove organic matter and phenolic compounds [14]. On the other hand, in order to work under sterile conditions, microfiltration with 0.2 μm membrane was chosen to eliminate microorganisms (fungus, yeasts and bacteria), reduce turbidity and improve light penetration.

For real OMW, a bioprocess involving a physicochemical as primary and a biological as secondary treatment (tested separately with lab equipment) was designed. The physicochemical treatment consisted of flocculation plus microfiltration units. Biological treatment was based on *S. obliquus* growth in different dilutions of industrial OMW as culture media. For this proposed process in its approach, it was considered the operational ease in its execution and operation. Low operational costs were achieved due to the natural sedimentation-flocculation without the addition of chemical compounds, only a small concentration of low-price flocculant was used. In addition, this process includes the production of algal biomass, which is not usually included in other conventional treatment processes.

3.1.1. Effect of primary treatment on wastewater characteristics

OMW composition before and after flocculation and microfiltration was determined with the aim to establish the nutrient removal by each operation.

In primary treatment total solids were notably reduced, resulting in the decrease of inhibitory compounds, turbidity and colour. In this sense, high reduction rates were achieved in the main parameters studied (Table 1).

Through flocculation, results showed that conductivity, turbidity, IC, COD, TPCs, TN and orthophosphate were reduced by 34%, 92.5%, 90.2%, 57.5%, 98.7%, 52.8% and 49.7%, respectively. The aim of this stage was to separate and reduce the total solids and total suspended solids content, determined in terms of turbidity. Despite TOC concentration which was increased from 328 mg/L to 530 mg/L. This fact may be due to the flocculant residue in treated OMW. In the same way, a decrease in the concentration of iron (7.56%), chloride (43.1%), sulphate (73.5%) and sodium (17.1%) was also determined.

In microfiltration unit, the following reduction percentages were registered: 98%, 82.6%, 13.8%, 85.1%, 22.7%, 39.1%, 15.1%, 36.6%, 93.6% and 1.84% for turbidity, COD, TPCs, TOC, TN, iron, chloride, sulphate, sodium, and orthophosphate, respectively.

The primary treatment proved to be effective in the reduction of most wastewater parameters. Flocculation could be highlighted as the most effective stage in terms of some of the most harmful compounds for microalgae growth, such as phenols and chloride. The presence of phenols in the culture medium results in inhibition for microalgal growth and smaller cell size [43]. The establishment of a primary treatment based on flocculation and microfiltration in the new proposed bioprocess is essential due to the role of flocculation in the removal of turbidity and OMW discoloration, allowing a greater light penetration in the culture. Microfiltration allowed higher removal rates of organic matter and iron, which at high concentrations can inhibit *S. obliquus* growth.

3.1.2. Secondary treatment based on Scenedesmus obliquus culture

Fig. 1A shows the variation of the biomass concentration through the experiment time for the 75% OMW culture. In all experiments with OMW ≥50%, a higher adaptation of *S. obliquus* to the culture media was observed by showing an abrupt increase (Lag phase, Fig. 1A) in the biomass concentration during the first 3 h of culture. This fact may be due to the higher availability of one or more essential nutrients.

In the exponential growth phase microalgae have a balanced growth due to the available nutrients in the culture medium and thus, cells divide at a constant rate depending upon the culture media composition and operating conditions, which results in biomass accumulation. The

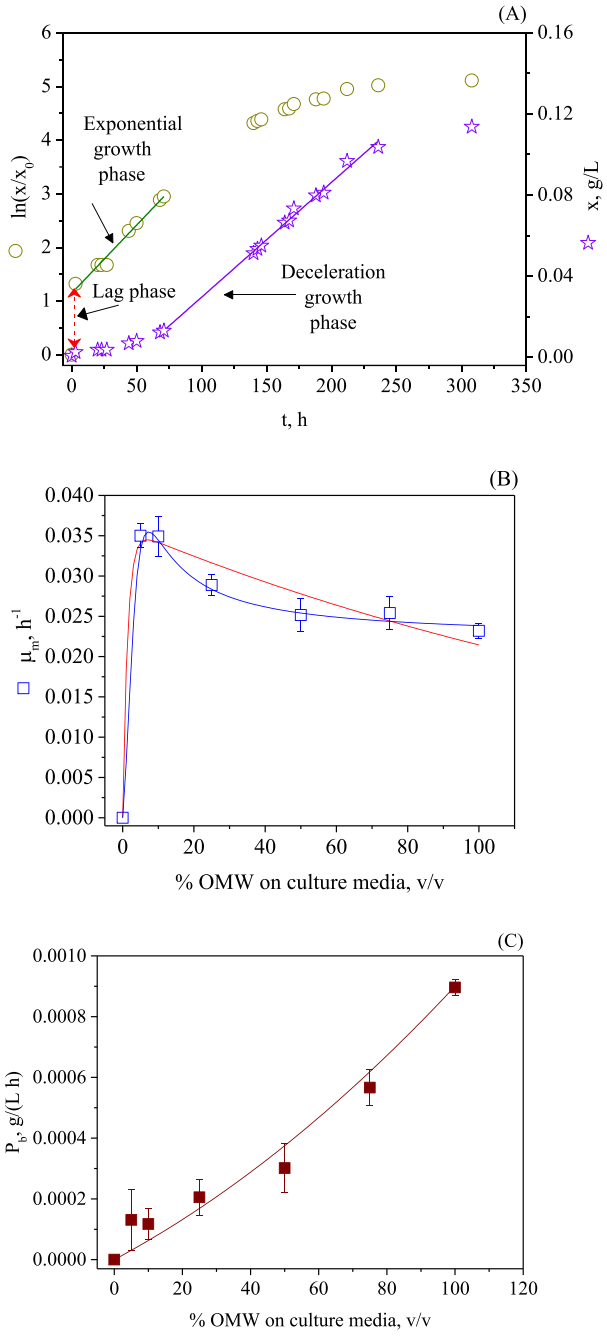

Fig. 1. A) *Scenedesmus obliquus* growth curves on 75% OMW. B) Maximum specific growth rates (μ_m) variation versus different OMW dilutions as culture media (Red and blue solid lines correspond to Teissier model [44] and Hodaifa et al. [45], respectively). C) Volumetric biomass productivities (P_b) variation versus different OMW dilutions as culture media (⟵ Solid line corresponds to simple second order equation model). Common operational conditions: agitation rate = 3.33 Hz, T = 25 °C, aeration rate = 0.5 min^{-1} and continued illumination intensity = 359 μE m^{-2} s^{-1}. Error bars represent standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

duration of this phase ranged from 19 h (25% OMW) to 72 h (100% OMW). The longest exponential phase in 100% OMW medium is due to the higher availability of essential nutrients at higher OMW concentrations.

The maximum specific growth rate, μ_m, was determined during the exponential growth phase according to equation (1),

$$ln\left(\frac{x}{x_o}\right) = \mu_m\, t + a \quad (1)$$

where 'x, g/L' is the biomass concentration at any time of the experiment, 'x_o, g/L' is the biomass concentration at the beginning of the experiment (t = 0 h), 'μ_m, h^{-1}' is the slope of the line and corresponds to the maximum specific growth rate, 't, h' is the time and 'a' is the intercept.

Fig. 1B shows that μ_m values were increased at lower OMW concentrations (μ_m = 0.035 h^{-1} in 5% OMW) and decreased (μ_m=0.0232 h^{-1} in 100% OMW) when the OMW concentration in the culture media was ≥50%. This behaviour may be due to the presence of inhibitory compounds (as residual oil) or light limitation by the increase of culture colour with the augment of OMW concentration in the culture media. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

After studying various inhibition and toxicity growth models by substrate, two of them reproduced the experimental variation of μ_m with %OMW concentrations. The first corresponds to the mathematical model of Teissier [44], Eq. (2),

$$\mu_m = \mu_{m,max}\left[e^{-S_0/K_I} - e^{-S_0/K_s}\right] \quad (2)$$

where '$\mu_{m,\ max}$ = 0,036 h^{-1}' is the maximum theoretical value determined for the maximum specific growth rate obtained, S_0 is the percentage of OMW in culture media, K_I = 193% is the value of the inhibition constant and K_S = 1.39% is the value of the slope for ½ $\mu_{m,\ max}$. The parameters of the goodness of the fit were r^2 = 0.964 and residual sum squares (RSS) = 2,46 × 10^{-5}.

The second model corresponds to the mathematical model proposed by Hodaifa et al. [45], Eq. (3),

$$\mu_m = \frac{\mu_{m1}\, K_s S_0 + \mu_{m2} S_0^2 + \mu_{m3} K_i K_s}{K_i K_s - K_i S_0 + S_0^2} \quad (3)$$

where S_0 is the percentage of OMW, μ_{m1} = 0.04 h^{-1} would correspond to the previously described $\mu_{m,\ max}$, μ_{m2} = 0.0223 is a constant value for μ_m at the highest %OMW (100% OMW), μ_{m3} = 1086 × 10^{-6} is a constant value for μ_m in the absence of OMW in the culture medium at S_0 = 0, K_S = 2.56% and K_I = 7.77%, which is the value at which the inhibition appears. The parameters of the goodness of the fit were r^2 = 0.996 and RSS = 3.96 × 10^{-6}.

In view of the results, it can be concluded that the $\mu_{m,\ max}$ = 0.036 h^{-1} obtained in the Teissier model [44] is lower than that obtained by Hodaifa et al. [45], μ_{m1} = 0,04 h^{-1}, since this value corresponds to the theoretical value without inhibition. The optimal value of μ_m was determined when %OMW was equal to 7.77% and 7.07% for Hodaifa et al. [45] and Teissier model [44], respectively. However, Hodaifa et al. [45] is the model that best fits the experimental behaviour since K_I = 7.77% is consistent with that observed experimentally in contrast to the value determined by Teissier model [44] (K_I = 193%).

In all experiments, a deceleration growth phase with linear behaviour was observed (Fig. 1A). In this phase of growth, the volumetric biomass productivity was calculated according to Eq. (4),

$$x = P_b\, t + b \quad (4)$$

where 'P_b, mg/(L h)' is the line slope and corresponds to the value of volumetric biomass productivity and 'b' is the intercept.

Fig. 1C shows the P_b values tendency. Data were fit to a second-degree polynomial model (r^2 = 0.985). The maximum value registered was P_b = 0.896 mg/(L h) in culture with 100% OMW medium.

The appearance of this linear phase may be related to limited availability of CO_2 [46], light [47] or both, and these two components were provided at a constant rate to the culture media. CO_2 was supplied through the aeration of the culture medium at constant value equal to 0.5 min^{-1} and the incident light intensity supplied to the photoreactors surfaces was the same for all experiments and equal to 359 μE $m^{-2}s^{-1}$. In this sense, nitrogen is an essential nutrient and it varied among the cultures due to the OMW dilution. Nitrogen is essential in proteins, chlorophyll, DNA, etc., formation. Low nitrogen concentrations inhibited *S. obliquus* division, leading to decreasing microalgal biomass productivity. TN content in 5% OMW culture medium was equal to 1.44 mg/L in comparison with 21.5 mg/L in 100% OMW medium. This variation in the culture media presented limited availability of nitrogen. In addition, the duration of the linear phase ranged from 27.5 h (5% OMW) to 240 h (50% OMW), then it decreased to 168 h in the culture with 100% OMW. This behaviour is consistent with the hypothesis of nitrogen limitation. The decrease in the phase duration in cultures with OMW concentrations higher than 50% may be due to the light limitation caused by the increase in culture coloration. Several microalgal species such as *Chlorella vulgaris, Chlamydomonas reinhardtii* or *Scenedesmus subspicatus* have shown similar behaviour under nitrogen limitation conditions [48,49]. *C. vulgaris* showed prolonged growth under N-replete conditions and yielded 1.8 times higher final biomass in comparison with N-limitation conditions [49]. Similarly, *C. reinhardtii* and *S. subspicatus* exhibited restricted cell division when cultured at low N concentrations; among three nitrogen concentration conditions (high-N culture = 19.6 mg/L, intermediate-N culture = 3.0 mg/L and low-N culture = 0.8 mg/L), both strains showed the lowest biomass in the low-N medium and notably increased biomass generation under high N-conditions [48].

3.2. Culture medium effect on final biomass generation and its biochemical composition

The final biomass concentration at the end of the cultures ranged from 0.029 g/L (5% OMW) to 0.21 g/L (100% OMW). Although these concentrations are low, the main goal of this work is the OMW treatment and in parallel, microalgal biomass with added value is generated. Today, urban wastewater is treated and citizens pay the cost of this treatment within our drinking water bill. No operations units included in this bioprocess are expensive. In fact, in our upcoming research works, the microfiltration unit is removed from the bioprocess and this is performed in non-sterile conditions.

A ternary diagram was plotted (Fig. 2) to represent biomass biochemical composition (lipids, proteins and carbohydrates, the main microalgae cells components). In this diagram, it can be clearly observed that lower nitrogen concentration in culture media resulted in higher carbohydrate content (72.5% and 18.7% in 5% and 50% of OMW, respectively). Then, carbohydrate and lipid contents increased to 43.2% and 44.9% in culture with 100% OMW, respectively (Fig. 2). Microalgae have the ability to accumulate carbon into energy-rich compounds (carbohydrates and lipids) as a response of a growth stress [50,51]. These results could be therefore due to light limitation caused by the light attenuation because of medium coloration, which is greater with the increasing of %OMW and thus the expected variation [22].

In addition, this fact was confirmed by the influence of turbidity in the light reaching microalgae inside the bioreactor, since the turbidity values in input to microalgae after dilution were varied as following 1.22 FTU, 1.53 FTU, 1.89 FTU, 2.43 FTU, 3.40 FTU and 4.09 FTU for cultures with 5%, 10%, 25%, 50%, 75% and 100% of OMW, respectively.

Protein content showed the opposite trend to that observed for carbohydrates and lipids. The increase in nitrogen concentration (1 mg/L to 10 mg/L) implied a protein content augment (from 4.65% to 64.2%). Then, protein content decreased to 10.8% in the culture with 100%

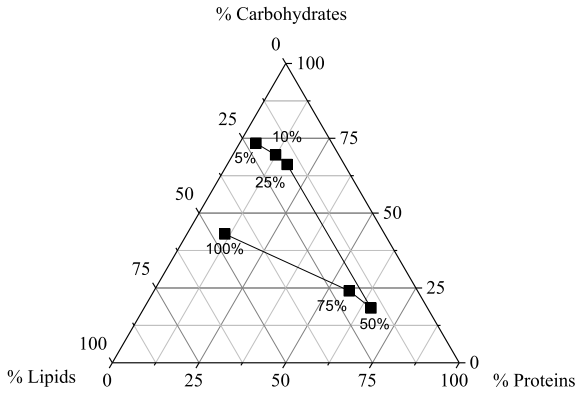

Fig. 2. Biochemical composition (percentages in dry weight of lipids, carbohydrates and proteins) of *S. obliquus* represented as ternary plot illustration for all cultures studied (5, 10, 25, 50, 75 and 100% OMW). Common operational conditions: agitation rate = 3.33 Hz, T = 25 °C, aeration rate = 0.5 min^{-1} and continued illumination intensity = 359 μE m^{-2} s^{-1}.

OMW (Fig. 2). This reduction may be due to the nutrient limitation as a result of an oil layer on the cells surface which blocked nutrients access, since higher OMW percentage in culture media implies high residual olive oil in the culture medium [45]. Nitrogen and phosphorous are essential constituents in protein structure and its synthesis is also related to both nutrients in the culture media.

Table 2 shows the fatty acids contents determined in the lipid fractions of algal biomass. These fatty acids are grouped into saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA). Fatty acids concentration is influenced by operating parameters as light intensity, nutrient availability, pH and temperature [51,52]. In the experiments, the last two parameters were kept constant and thus the variability in fatty acid profiles could be attributed to nutrients availability and light intensity. In this sense, the following fatty acid percentages were registered: saturated (51.1%–64.1%), monounsaturated (22.6%–37.5%), polyunsaturated (0.17%–0.18%) and the sum of saturated plus monounsaturated (86.8%–98.9%). Among the saturated fatty acids, the most abundant was palmitic acid (42.3%–54.8% of C16:0), followed by stearic acid (6.18%–7.10% of C18:0) and among the monounsaturated, the most abundant was oleic acid (21.4%–35.1% of C18:1n9). The high saturated and monounsaturated fatty acid percentages obtained (86.8% and 98.9%, respectively) are the most suitable components for high quality biodiesel production since they contribute to some important properties of biodiesel as density, viscosity, oxidative stability and heating value [53]. The only polyunsaturated fatty acid identified was linoleic (C18:2*n*6) at low concentrations (<1%) in the biomass obtained from culture media ≤25% OMW. High polyunsaturated fatty acids levels are not desired for biodiesel production due to their ease degradation and oxidation [54].

Table 2
Fatty acid profiles obtained on lipid fraction of *S. obliquus* biomass harvested at the end of the experiments.

% Fatty acids	Olive-oil mill wastewater concentration, %						CV*, %
	5	10	25	50	75	100	
C14:0	0.42	0.6	0.42	0.37	0.37	0.33	20.8
C16:1	2.15	1.24	11.4	2.15	1.26	2.4	104.6
C16:0	48.9	54.8	52.5	53.8	50.7	42.3	8.2
C18:2*n*6	0.17	0.18	nd	nd	nd	nd	2.9
C18:1*n*9	30.7	21.4	25.4	28.1	31.6	35.1	15.4
C18:0	6.49	6.23	6.62	6.18	7.10	6.56	4.6
C20:0	1.22	1.58	1.37	2.14	2.26	0.59	37.0
C22:0	0.4	0.51	0.46	0.41	0.41	0.37	10.7
C24:0	0.23	0.33	0.30	0.25	1.57	0.22	100.9
C26:0	0.14	0.17	0.22	nd	nd	0.16	17.1
C28:0	0.76	nd	0.17	0.97	0.79	0.63	40.6
ΣSFA**	58.7	64.2	62.1	64.1	63.2	51.1	7.6
ΣMUFA***	32.9	22.6	36.8	30.2	32.9	37.5	15.4
ΣPUFA****	0.17	0.18	nd	nd	nd	nd	2.9
ΣSFA+ ΣMUFA	91.6	86.8	98.9	94.3	96.1	88.6	4.5
Unidentified	8.23	13.0	1.10	5.70	3.90	11.4	57.3

*Coefficient variation = standard deviation*100/mean.
**Corresponding to the sum of saturated fatty acids.
*** Corresponding to the sum of monounsaturated fatty acids.
**** Corresponding to the sum of polyunsaturated fatty acids.

In general, the cultures with 10%–75% of OMW did not register a significant difference in the saturated fatty acid percentages (63.4 ± 0.85%). The difference determined in cultures with 5% and 100% may be due to the high unidentified fatty acids (8.23% and 11.4%).

The harvested biomass could have direct use in combustion or by its fractionation into lipids, carbohydrates and inert fractions. The first fraction could be destined to biodiesel production. The second in alcoholic production through anaerobic fermentation and the third could be used in anaerobic digesters for biogas production. All these possibilities allow the generation of energy, which could be transformed into different forms such as heat, fuel, and electricity. Although this biomass has nutritional value, the current legislation does not allow its use in human or animal feeding. In any case, the biomass represents a sustainable resource for energy production and a clean energy. In brief, this is an added value in form of energy alongside the wastewater treatment (main objective of this bioprocess).

3.3. Pollutants removal by *S. obliquus*

Microalgae can consume organic and inorganic nutrients from wastewaters for cell generation. This removal can be calculated by measuring the following parameters: TC, TOC, IC, TN, PO_4^{-3} and total iron ions.

3.3.1. Total organic and inorganic carbon removal

Fig. 3 (A and B) shows the variation of TOC and IC concentrations in OMW (without *S. obliquus* biomass) over the course of the experiments. For both concentrations of carbon species, a sharp decline in these values was observed during the first 27 h of the cultures, except in the case of 5% OMW culture. This descent matches with the exponential growth phase in which the maximum specific growth velocity was determined. In the subsequent growth phases, a slightly decrease in these values was observed. In the case of OMW without dilution (100% OMW), an increase in final TOC and IC values was registered due to the release of intracellular compounds from ruptures of dead cells [11,55].

TOC (−23.5%, 43.5%, 39.3%, 67.4%, 48.5% and 20.5%) and IC (73.2%, 76.8%, 88.5%, 95.8%, 50.1% and 40.2%) removal percentages were determined for 5%, 10%, 25%, 50%, 75% and 100% OMW cultures, respectively. The negative percentage indicates an increase in the final TOC values for 5% OMW culture.

The maximum removal values for TOC and IC were registered in the culture with 50% of OMW. However, the maximum specific growth velocities were determined in the cultures with 5% and 10% of OMW. These good results are due to the lack of toxic constituents at low OMW concentrations by dilution effect. In addition, this fact could be explained by considering that *S. obliquus* changed its metabolism from autotrophic (in uncoloured culture with 5% of OMW with virtually no TOC uptake (Fig. 3A)) to mixotrophic growth (in the cultures with OMW concentration among 10% and 50%) to heterotrophic cultures for the other cultures (75% and 100% OMW). The augment of OMW in the culture media increases cultures colour. Similar results were previously

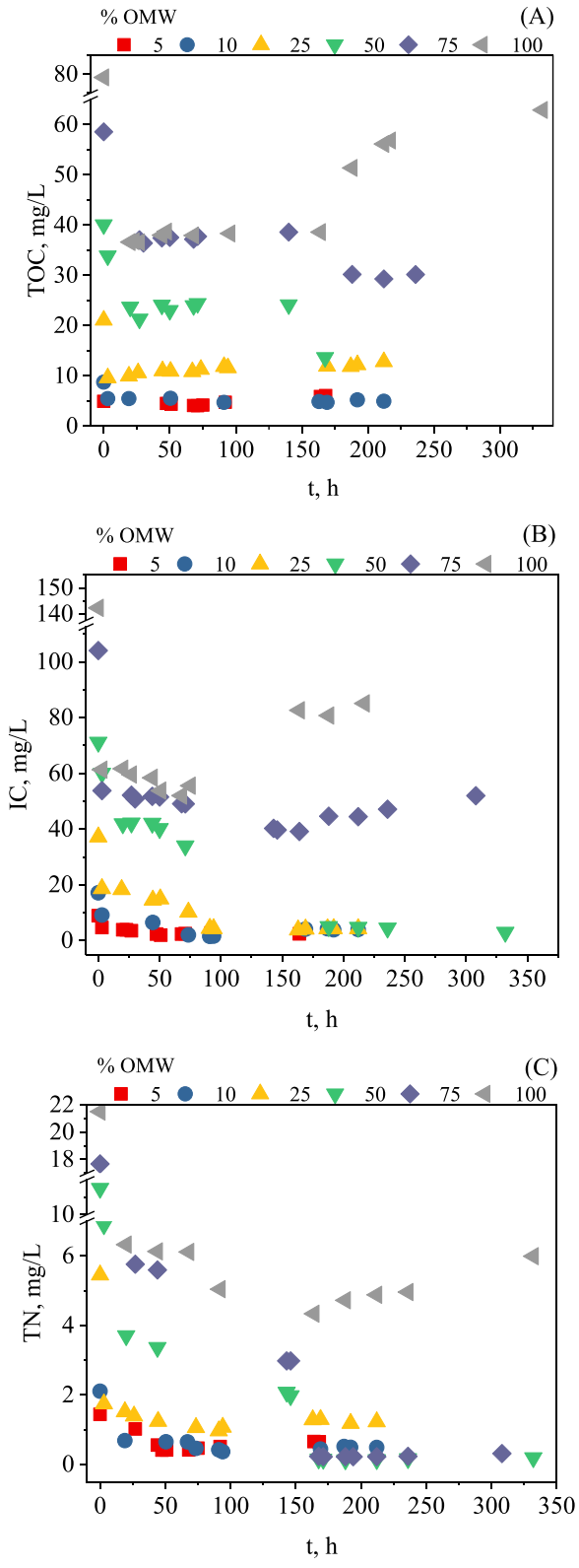

Fig. 3. Variation of total organic carbon, TOC (A), inorganic carbon, IC (B) and total nitrogen, TN (C) on the treated OMW dilutions (without algal biomass) along the cultures. Common operational conditions: agitation rate = 3.33 Hz, T = 25 °C, aeration rate = 0.5 min^{-1} and continued illumination intensity = 359 μE m^{-2} s^{-1}. The experimental data of TOC, IC and TN were determined at least twice with coefficient variation (CV) < 2 (Coefficient variation = standard deviation*100/mean).

showed in our work, demonstrating that high fat matter and colour in undiluted OMW act as limiting factors for *S. obliquus* growth and nutrients uptake [56].

3.3.2. Total nitrogen removal

Total nitrogen of OMW (without *S. obliquus*) variation throughout the experiments is shown in Fig. 3C. It can be observed, in all experiments, a steeper decrease during the first hours of cultivation, which corresponds with *S. obliquus* exponential growth phase. Then, nitrogen uptake slightly decreased and remained virtually constant at the end of the culture. Global total nitrogen reduction was equal to 54.8%, 76.8%, 77.5%, 98.2%, 98.2% and 72.1% for culture media with 5%, 10%, 25%, 50%, 75% and 100% OMW, respectively. These removal percentages are consistent with protein concentration determined in final biomass generated. Highest protein contents 64.2% and 55.4% were achieved in cultures media with 50% and 75% of OMW, respectively. Lower nitrogen availability (1.44 mg/L) in 5% of OMW culture resulted in minor biomass and protein generation 0.029 g/L and 4.65%, respectively. In this sense, nitrogen disposal must be controlled since excess nitrogen lead to eutrophication water bodies [8].

3.3.3. Total phenolic compounds removal

Fig. 4A shows the variation of TPCs concentration in OMW over the course of the experiments. In the 100% OMW culture, the TPCs removal was performed in two steps. In a first step, a pronounced decrease during approximately the first 50 h of the culture was observed. Then, a slow decrease with linear behaviour. On the other cultures, it can be considered a linear behaviour (zero-order equation model) for TPCs concentration throughout the experimental time, since the initial TPCs concentrations in these cultures (5%–75% of OMW) were less than 3 mg/L. In this way, TPCs final concentrations below 1 mg/L were achieved in culture media containing ≤25% OMW. In any case, it is important to point out that only small consumption of phenolic compounds was expected since phenolic compounds are toxic for microalgae.

Fig. 4B shows TPCs removal rates and final global removal percentages obtained in the different culture media studied. The highest removal TPCs rate values (−0.00106 and −0.00160 mg/(L h)) and elimination percentages (54.4% and 59.1%) were obtained in cultures with 5% and 10% of OMW. Cultures with OMW percentages equal or higher than 25% registered similar removal percentages around 35%. The removal percentages of TPCs tendency shows an inhibition effect of phenolic compounds at higher OMW concentrations.

Several studies have shown the ability of different microalgae strains to remove phenols from wastewaters. Cheng et al. [57] proved that the oleaginous microalgae *Tribonema minus* was able to efficiently degrade phenols from an initial concentration in the culture media of up to 700 mg/L and this TPCs biodegradation was directly influenced by the initial concentration of TPCs in the medium. In this work, the maximum phenol removal percentage was equal to 94.6% at an initial phenol concentration of 250 mg/L. Lee et al. [58], indicated that *Spirulina maxima* is able to grow on synthetic wastewater culture media with phenols up to 400 mg/L, achieving a 97.5% of phenol removal. Furthermore, Stephen and Ayalur [59] obtained high phenols removal levels (91%) when growing *Chlorella pyrenoidosa* on a phenolic effluent of a coal gasification plant (20% of effluent). In this study, the phenolic compounds in the culture media were varied from 282 mg/L to 846 mg/L.

Finally, according to APHA [60] all treated OMW could be directly discharged into public sewers, with a permissible limit of phenols equal to 5 mg/L. However, cultures with 5%, 10%, 25% and 50% are suitable for discharge into inland surface waters, with an admissible limit of 1 mg/L. In general, all treated OMW could be discharged into inland surfaces waters and public sewers since the final TPCs concentration are remarkably close to the lowest value required.

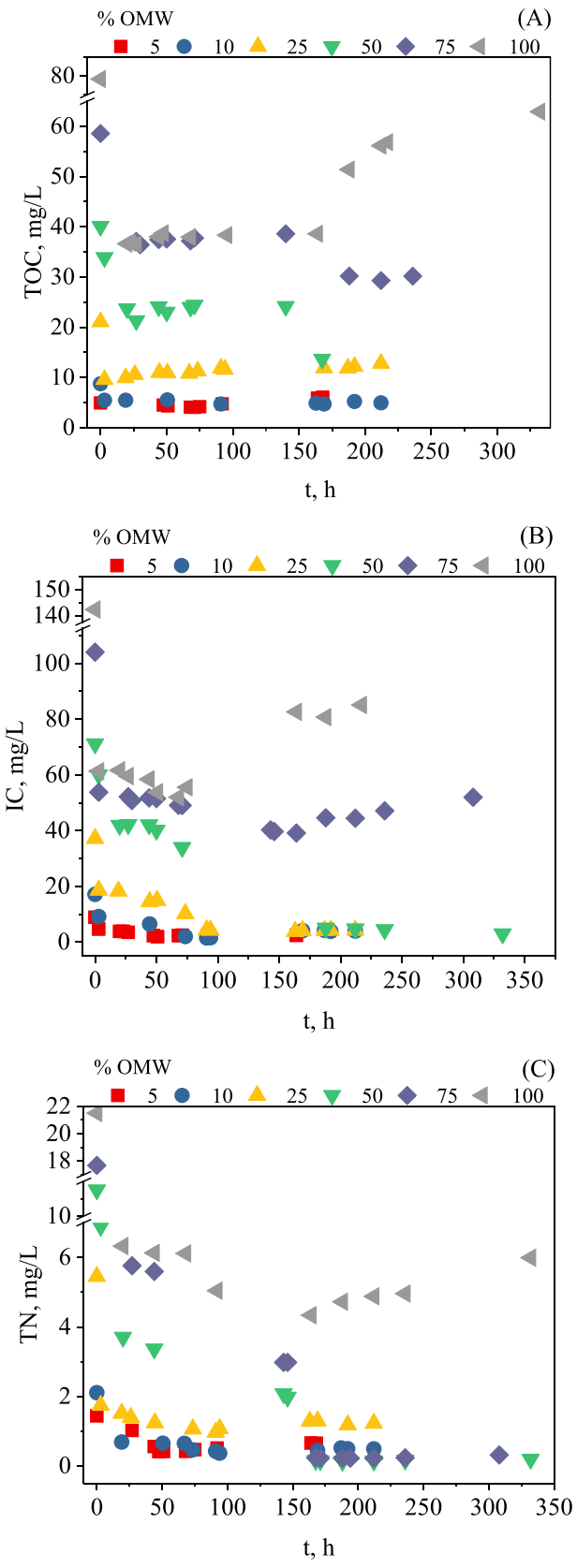

Fig. 4. A) Variation of total phenolic compounds (TPCs) concentration in OMW along the cultures. B) Total phenolic compounds removal velocities and final TPCs removal percentages. Common operational conditions: agitation rate = 3.33 Hz, T = 25 °C, aeration rate = 0.5 min^{-1} and continued illumination intensity = 359 μE m^{-2} s^{-1}. Error bars represent standard deviation.

3.3.4. Reduction on minority compounds

Orthophosphate and total iron as minor compounds were measured at the beginning and the end of the experiments, since orthophosphate have a key function in the synthesis of proteins, nucleic acids and phospholipids and iron is a crucial element in photosynthesis and respiratory transport chains of electrons. The orthophosphate removal percentages in OMW were ranged from 53.7% to 70.2% in cultures with 75% and 5% of OMW, respectively.

Iron removal percentages ranged from 5.91% to 46.1% in cultures with 75% and 25% of OMW. The consumption of this element by *S. obliquus* is due to that iron improves the photosynthetic activity and increases the biomass productivity [31].

4. Conclusions

The combination of a physicochemical treatment (primary treatment) based on flocculation and microfiltration plus microalgal growth of *S. obliquus* culture (secondary treatment) has been established for the treatment of industrial OMW. This combined process allowed the wastewaters treatment and the generation of a valuable microalgae biomass. Primary treatment allowed high global removal levels of organic and inorganic matter, which resulted in a culture media with less turbidity, colour and colloidal particles, favouring culture illumination. As a result of the previous treatment, algal growth registered maximum specific growth rate (μ_m = 0.035 h^{-1}) and biomass productivity (P_b = 0.896 mg/(L h)) in cultures with 5% and 100% of OMW, respectively. In addition, high removal percentages up to 67.4% (50% OMW), 95.8% (50% OMW), 98.2% (50% OMW) and 59.1% (10% OMW) were determined for TOC, IC, TN and TPCs, respectively. On the other hand, the final biomass obtained was rich in energetic compounds, with maximum carbohydrate and lipid contents up to 72.5% (5% OMW) and 44.9% (100% OMW), respectively.

The scale up of the industrial OMW treatment could be established as a combination of physicochemical (flocculation and microfiltration) and microalgal treatments (*S. obliquus* culture). For biodiesel production, the best operating conditions to apply are: OMW without dilution, aeration rate 0.5 min^{-1}, agitation speed 3.33 Hz, continuous illumination, and temperature equal to 25 °C. In these conditions, highest biomass (0.21 g/L) and lipids (44.9%) generation were obtained. From the point of view of pollutants removal, the use of a culture medium with 50% of OMW resulted in the following removal percentages: TOC 67.4%, IC 95.8%, and TN 98.2%. For phenolic compounds removal, the highest removal velocities (−1.06 μg/(L h) and −1.60 μg/(L h)) and percentages (54.4% and 59.1%) were determined in the culture media with 5% and 10% of OMW, respectively. In any case, in a real process, temperature and illumination would be variables imposed by natural conditions, which means that solar light and ambient temperature would be used.

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CULTIVATION OF *Scenedesmus obliquus* IN MIXTURES OF URBAN AND OLIVE-OIL MILL WASTEWATERS FOR THE DUAL APPLICATION OF ALGAL BIOMASS PRODUCTION AND WASTEWATER TREATMENT

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ABSTRACT: Olive-oil mill wastewater (OMW) is a great environmental problem because of its high organic load plus another antioxidant compounds as phenolic compounds. On the other hand, the treated urban wastewater (TUW) in depuration plants, which have primary and secondary treatment processes, is directly disposed to public waterways. Both wastewaters could be used as sources for microalgal culture media constitution. These wastewaters are rich in nitrogen and phosphorus compounds such as ammonium, nitrates and phosphates as well as organic and iorganic compounds. The revalorization of these wastewaters throughout the microalgal biomass production and the reutilization of the final treated water were studied. The crude OMW was pretreated by flocculation and ultraviolet light before microalgal culture. All microalgal experiments were done in batch photo-bioreactors (1 L work capacity) at laboratory scale. The operational conditions were agitation rate = 200 rpm, T = 25 ºC, aeration rate = 0.5 L/min and continuous light with illumination intensity equal to 359 μE m^{-2} s^{-1}. Results revealed that the new proposed process lead to improve the final water quality. High removal percentages of organic matter and nitrogen species were registered. The final biomass obtained was characterized by high energetic compounds percentages (carbohydrate and lipid contents).

1 INTRODUCTION

One of the major concerns that industries must face is the large amount of wastewater that are generated as a consequence of their activity. In addition to industrial effluents, huge quantities of urban wastewaters (UW) are generated by industrialized countries [1]. This substantial volume of residual waters have to be treated to avoid environmental contamination and to ensure public health with safe water supplies [2]. In addition, according to the World Health Organization (WHO), freshwater scarcity is a matter that will affect > 40% of the world's population in the next 50 years [3]. To solve these problems, new methods for wastewater treatment must be explored in order to get suitable water for reuse in irrigation, discharge to receiving waters or for being reused in the same industries where they are generated [4].

Between the different treatment processes for residual waters, bio-treatment with microalgae is particularly attractive since microalgae are photosynthetic microorganisms which convert solar energy into useful biomass and incorporate nutrients such as nitrogen or phosphorus from the effluents [5]. In addition, microalgae present many other advantages such as ease of cultivation since they can grow almost anywhere with little attention using unsuitable water for human consumption [6]. Its use as a wastewater treatment requires the proper selection of the microalgae specie with a series of specific characteristics such as high growth rate, high lipid content and productivity and a large tolerance to pollutant compounds such as metal ions, pathogenic microorganisms or phenolic compounds among many other components which can harm microalgae growth and are extensively present in different wastewater streams [7].

Wastewaters can be classified in several categories such as municipal, pharmaceutical, agro-industrial or textile dyes wastewater among many others [7]. Each type has its own physicochemical characteristics as well as its own nutrient composition and presence of potential inhibitors [8,9]. These effluents require a treatment before being dumped into rivers, lakes or the sea in order to achieve environmentally safe levels of the contaminants present in their composition (ammonium, nitrates, phosphates, etc.), which can contribute to the eutrophication of the receiving effluents [1].

Urban wastewaters (UW) are generated as a combination of water and wastes from homes, commercial and industrial facilities. UW are characterized by containing high concentrations of toxic compounds, organic matter, pathogenic microorganisms etc. [8]. On the other hand, olive-oil mill wastewater (OMW) is a secondary product generated during the olive oil extraction process characterized by its dark brown color, strong odor, acid pH as well as high values for the most polluting parameters: biological and chemical oxygen demand (BOD and COD, respectively), phenolic compounds, nitrogenous compounds [9] as well as tannins, pectins, lignins, fatty acids etc. [10].

In this work, the use of UW as well as mixtures with OMW as culture medium for *Scenedesmus obliquus* were studied. The proposed process consisted of a primary treatment, based on a physico-chemical treatment, followed by a biological treatment performed by the microlgae. The primary treatment proposed consisted of the flocculation-sedimentation, photolysis by artificial UV light and microfiltration (0.2 μm membrane size). In all cases a real raw OMW and UW were used. To achieve the aim of this work, physico-chemical characteristics of both wastewaters, biomass production and its biochemical composition were determined. From experimental results obtained the kinetic growth parameters were calculated. Final treated water quality and its reuse were established.

2 EXPERIMENTAL

2.1 Microorganism and culture conditions

The freshwater microalgae used was *S. obliquus* CCAP 276/3A which was supplied by the Culture Center for Algae and Protozoa, Oban (UK). Experiments were carried out in sterile conditions, on a laboratory scale in stirred batch tank reactors with illumination on frontal

side and the following characteristics of each reactor: working capacity = 1 L, diameter = 10 cm and height = 16 cm.

2.2 Experimental procedure

UW was obtained from a conventional primary and secondary-treatment plant located in Seville (Spain) as well as the OMW, obtained from an olive oil extraction plant from the same province in which oil is extracted by the two-phase centrifuge process.

Mixtures of OMW and UW, previously filtered and sterilized through a membrane with 0.2 μm pore size were used as culture media. Prior to the preparation of the mixtures, the flocculation-sedimentation, photolysis and microfiltration of the raw OMW was performed.

The flocculation-sedimentation had a duration of 90 min. An Imhoff funnel and a commercial flocculant Flocudex CS-51 were used in this stage (concentration = 1 g/L). The photolysis was performed in a batch stirred photoreactor with total capacity equal to 750 cm3 (work volume = 600 cm^3). A commercial medium pressure UV immersion lamp, model TQ 150 Brand HNG Germany G4, 150 No 5600 1725 was used.

For the culture media preparation, the OMW concentrations added to raw UW were 0%, 5% and 10% (v/v). The common culture conditions were: temperature = 25ºC, pH = 7, aeration rate = 0.5 L min^{-1}, pH value = 7, magnetic agitation speed = 200 rpm and continuous light with illumination intensity equal to 359 μE m^{-2} s^{-1}.

S. obliquus inoculum consisted of a preculture in Rodríguez-López [11] mineral synthetic medium solidified with agar at 2% (v/v) and incubated for seven days under continuous illumination at room temperature. Cells were transferred to the sterilized culture medium after resuspension in sterilized ultrapure water.

2.2 Analytical methods

Biomass concentration was determined through the measurement of the absorbance of the cell suspension in ultrapure water at 600 nm.

The characterization of crude and treated wastewaters was performed through the determination of the following parameters: pH value, electric conductivity, turbidity, total phenolic compounds (TPCs), chemical oxygen demand (COD), total carbon (TC), total organic carbon (TOC), inorganic carbon (IC), total nitrogen (TN), total iron (Fe), chloride (Cl^-) and sulphates (SO_4^{-2}) (Hodaifa et al., 2015). Sodium (Na^+), ammonium (NH_4^+), calcium (Ca^{2+}) and potassium (K^+) were determined by Crison selective electrode, mod. GLP 22. Orthophosphate by Macherey-Nagel test (0.2-5 mg/L).

Biomass obtained at the end of the culture was separated by centrifugation at 3000 rpm for 5 min and washed three times with distilled water. After drying at 105 ºC, total lipids, proteins and fatty-acids contents were determined.

Total lipids were extracted in a micro-soxhlet apparatus using n-hexane as solvent. Fatty acid profile was determined and identified by gas chromatography using a Hewlett–Packard, Model 5890 Series II equipped by a FID detector. The crude protein content was calculated after the determination of total nitrogen concentration using a Total Carbon and Nitrogen Analyzer provided by Skalar Company, mod. Formacs^{HT} and Formacs^{TN} according to the following equation: %Crude proteins = %TN×6.25.

The total carbohydrate content was obtained by considering that algal biomass is formed by proteins, carbohydrates, lipids, pigments and genetic material (considered approximately about 1%).

3 RESULTS

3.1 The wastewaters

Table 1 shows the physical and chemical parameters of raw OMW and UW used in the formation of the culture media in the different experiments, as well as their composition after the primary treatment. The raw OMW was also characterized before flocculation and UV photolysis as follows: conductivity = 1.9 mS/cm, turbidity = 714 FTU, COD = 5839 mg O_2/L, TPCs = 322 mg/L, TC = 1400 mg/L, TOC = 646 mg/L, IC = 318 mg/L, TN = 58.9 mg/L, NH_4^+ = 4.44 mg/L, SO_4^{-2} = 320.3 mg/L, PO_4^{-3} = 43.1 mg/L, Na^+ = 0.943 mg/L and Fe = 1.19 mg/L. In this sense, for the use of wastewaters as culture media for microalgae, it must contain a proper nutrient profile, being carbon, nitrogen and phosphorous sources the most essential components for microalgal biomass generation.

Table I: Characterization of the initial raw UW and treated OMW (flocculation, photolysis and micro-filtrated) used as culture media for *S. obliquus*.

Parameter	Raw		OMW+UW mixtures after micro-filtration (%OMW in UW)		
	OMW**	UW	0	5	10
EC, mS/cm	1.99	1.32	1.47	1.39	3.46
Turbidity,FTU	32.9	26.3	2.18	0.90	5.10
COD, mgO_2/L	3746.5	109.9	74.5	227.1	319.3
TPCs, mg/L	21.04	0.22	0.04	0.33	0.75
TOC, mg/L	371.9	22.1	3.37	30.7	150.8
TC, mg/L	426.1	48.05	65.5	71.6	159.6
IC, mg/L	54.1	25.9	59.2	40.9	8.72
TN, mg/L	6.56	6.99	20.8	7.61	5.00
NN, mg/L*	-	0.57	6.98	0.73	-
NH_4^+, mg/L	0.07	0.002	0.07	0.76	-
Cl^-, mg/L	580.1	602.02	246.1	286.3	300.3
SO_4^{-2}, mg/L	1276	578.7	666.8	701.8	869.3
PO_4^{-3}, mg/L	26.8	0.40	0.21	1.35	0.33
K^+, mg/L	24.4	2.30	1.73	18.4	-
Na^+, mg/L	-	-	2.00	-	-
Ca^{2+}, mg/L	26.4	33.7	996.4	0.78	-
Fe, mg/L	0.71	0.48	-	0.28	3.09

**NN is the sum of NO_3+NO_2.*
***OMW treated by flocculation and photolysis with artificial UV light.*

With respect with the OMW treated by flocculation and artificial UV light it must be highlighted it is high organic load, determined in terms of: turbidity = 32.9 FTU, COD = 3746.5 mg O_2/L, TPCs = 21 mg/L and TOC = 371.9 mg/L. Nevertheless, TN = 6.56 mg/L indicates a N deficiency in OMW. The presence of ortho-phosphate (26.9 mg/L) in the culture media plays an important role in microalgae cell growth and metabolism through phosphorylation reactions [12]. High chloride (Cl^- = 580.1 mg/L) and sulphate ($SO4^{-2}$ = 1276.2 mg/L) concentrations were detected. These two last compounds can harm microalgae growth since they are highly inhibitory of microalgae growth. High iron concentration are not desired, the low concentration detected in raw OMW can be explained by the use of drinking water in food industries for washing raw materials [12]. All these

organic and inorganic nutrients can be used by microalgae to generate biomass.

With respect to raw UW it must be highlighted the high presence of chloride (Cl^- = 602.02 mg/L) and sulphate ($SO4^{-2}$ = 578.7 mg/L), which can inhibit microalgae growth. Nevertheless, phenolic compounds and iron, which are greatly toxic for microalgae were found in low concentrations, 0.22 and 0.48 mg/L, respectively. In general, high levels of organic matter were not found: turbidity = 26.3 FTU, COD = 109.9 mg O2/L, TOC = 22.1 mg/L and TN = 6.99 mg/L. Low concentrations of phosphorus in the form of inorganic salts (ortho-phosphate = 0.40 mg/L) were also found.

Physicochemical characteristics of wastewaters resulting from the mixtures of UW and OMW are also recorded in Table 1. In view of the results it can be concluded that the addition of a higher proportion of OMW lead to an increase in most of the parameters studied, more significantly in the organic load. Higher levels of chloride, sulphate and iron were also found in the mixture composed by 10% OMW (v/v).

The efficient growth of microalgae in wastewater is influenced by several factors such as temperature, pH, light availability and concentration of essential nutrients such as N, P and organic C among many others [8]. For this reason, wastewaters containing high organics, nitrogen and phosphorus sources have a higher potential towards microalgae cultivation and simultaneously microalgal wastewater treatment. For this reason, the supplementation of UW, with low organic load, with OMW, which contains a higher organic matter concentration could lead to an improvement of microalgal growth. In addition, the higher concentration of TN in the UW could also enhance microalgal growth, since nitrogen is one of the major nutrients required for micralgae cultivation, as it constitutes about 1-10% of the microalgal biomass [13]. The variation of OMW and UW may allow the development of a complete culture medium with all the nutrients required for microalgae growth [8].

3.2 *Scenedesmus obliquus* growth

Fig. 1 shows a sample of the growth curves of *S. obliquus* when the microalgae was grown in a culture media composed by 5% OMW (v/v) and 95% UW (v/v). In none of the experiments a lag phase was observed at the beginning of the culture. Adaptation phase is a period in which microalgae get adapted to a new environment, this phase must be as short as possible in order to improve biomass productivity [14].

In all the experiments, the exponential was the first growth phase observed with a duration which ranged from 167 (10% OMW (v/v)) to 235 h (5% OMW (v/v)). This phase is characterized by the availability of all nutrients required for microalgal biomass accumulation, with carbon, nitrogen and light as the most essential compounds [14].

The determination of the maximum specific growth rate of *S. obliquus* was done during this phase according to equation (1),

$$\ln (x/x_o) = \mu_m t + a \quad (1)$$

where 'μ_m' is the slope of the line and corresponds to the maximum specific growth rate and 'a' is the intercept.

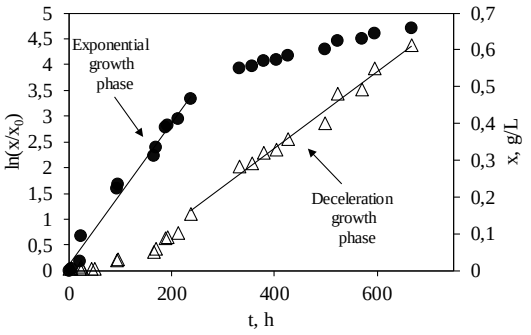

Figure 1: Graphical determination of maximum specific growth rate and volumetric biomass productivity. Operating conditions: Culture medium = 5% OMW and 95% UW, agitation rate = 200 rpm, T = 25 ºC, aeration rate = 0.5 L/min and illumination intensity = 359 μE m^{-2} s^{-1}.

The highest value of μ_m was achieved when a 100% UW culture media was used (0.0202 h^{-1}), followed by the mixture in which 5% OMW (v/v) was added to raw UW (0.0138 h^{-1}) and by last, culture media with 10% OMW (v/v) added to raw UW (μ_m = 0.0122 h^{-1}).

A phase of linear increase in biomass with time was observed after the exponential phase with a duration which ranged from 22.5 (100% UW (v/v)) to 431 h (5% OMW (v/v)). This phase is determined by the limitation of one or more nutrients such as CO_2 or light. In all experiments the CO_2 supply was performed through constant aeration with air at 0.5 v/v/min and light intensity was constant and equal to 359 μE m^{-2} s^{-1}.

Productivity of *S. obliquus* was determined during the deceleration (linear) growth phase using equation (2),

$$x = P_b t + a \quad (2)$$

where 'P_b' is the slope of line and corresponds to the volumetric biomass productivity and 'b' is the intercept.

Similar values of biomass productivities were obtained in all experiments, ranging from 1.0 (5% OMW (v/v) to 1.2 mg/(L h) (100% UW (v/v)) .

Finally, a stationary phase as well as the onset of cell death was observed at the end of some experiments. This phase is related to nutrients-starvation conditions. After reaching a peak point in microalgae biomass concentration, this phase is characterized by the accumulation of intracellular energy-storage compounds [14].

Fig. 2 shows the variation of all carbon (A) and nitrogen (B) species concentrations with time in the microalgal biomass. It can be observed in Fig. 2A a TC and TOC increase in biomass along the culture explained by the algae's ability to capture C from the culture medium and fix it and incorporate it into biomass structures, which resulted in an increment of the TC and TOC concentrations in the biomass along the culture. IC levels in the biomass showed a little, almost negligible, rise along the culture.

It can also be observed in Fig. 2B a rapid increase in TN during the starting period, particularly in the first 200 h, corresponding this increment with the exponential growth phase of the microalgae. This proved that nitrogen consumption was associated with microalgal growth and its conversion into biomass structures, mainly proteins. Once *S. obliquus* growth was stopped, the

concentration of TN in the biomass remained constant until the end of the culture. No nitrate-nitrite was found in the biomass.

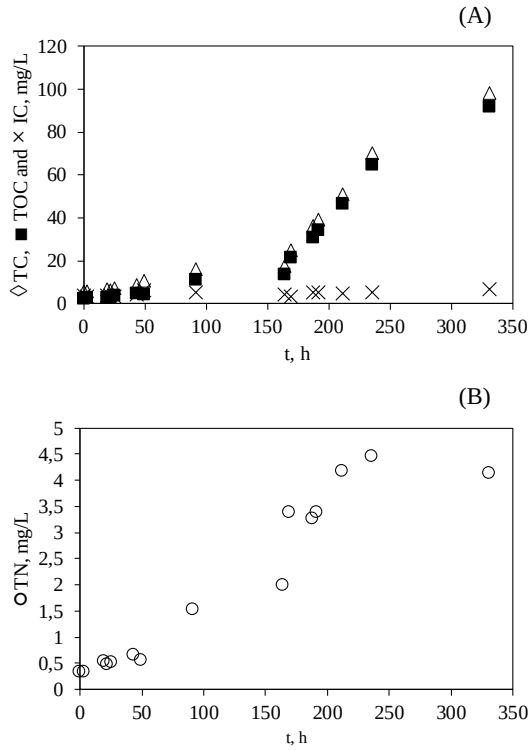

Figure 2: Variation of total carbon species (A) and total nitrogen (B) on *Scenedesmus obliquus* biomass during the culture.

3.3 Biochemical composition of *S. obliquus* biomass

The biochemical composition of the biomass at the end of the experiments was influenced by the culture media composition. At the end of each experiment, the harvested biomass of *S. obliquus* was analyzed and the determination of proteins, carbohydrates and lipids contents was performed. In addition, the total pigments (total chlorophylls and total carotenoids) were determined along the cultures. These are the microalgae cells main components. The variation on the biomass composition of *S. obliquus* for all culture media studied is shown in Table 1.

Comparing the protein content obtained in the biomass under the different culture conditions it was found that the highest protein content was achieved when 100% UW was used (initial TN culture medium = 20.8 mg/L and protein yield = 57.7%). The main compound required by microalgae for protein synthesis is the nitrogen, for this reason, further nitrogen concentration in the culture media can lead to a further microalgae protein content. 4.06 % and 7.54 % protein yields were obtained in the cultures with 5% and 10% OMW (v/v), respectively.

These results are consistent with the lipid yields obtained. Microalgae tend to accumulate lipids under stress conditions, such as nitrogen starvation, the initial TN concentration in the culture media containing 5% OMW was equal to 7.61 mg/L, and initial TN in 10% OMW culture media was equal to 4.99 mg/L in comparison with initial TN availability = 20.79 mg/L in the 100% UW culture media, in which the lowest % lipid was obtained and equal to 3.16 % in comparison with the highest lipid content obtained equal to 19.7 % in the 10% OMW culture media. The obtaining of a high lipid fraction in the final biomass gives rise to the possibility of using this fraction for biodiesel production.

Carbohydrate content increased at lower nitrogen concentrations in the culture media, which is consistent with previous findings showing that carbohydrate accumulation in microalgae is triggered by nitrogen depletion [15]. 37.2%, 72.2% and 75.3% of carbohydrates were obtained in the 0%, 5% and 10% OMW (v/v) in UW cultures. These high values are also indicative of the nitrogen deficiency, which resulted in the accumulation by the microalgae of organic compounds such as polysaccharides. Biomass with high carbohydrates content is suitable for its use in biofuels generation [16].

In view of the biochemical composition results it can be concluded that *S. obliquus* is a versatile microalgae capable of adapting its biochemical composition to the culture media and the availability of nutrients.

Table II: Metabolites yields (% dry cell weight) of *Scenedesmus obliquus* final biomass.

%OMW in UW (v/v)	Proteins %	Lipids %	Pigments %	Carbohhydrates %
0	57.7	3.16	0.94	37.2
5	4.06	19.7	0.06	75.2
10	7.54	15.9	0.22	75.3

*Values calculated considering algal biomass formed by proteins, carbohydrates, lipids, pigments and genetic materials (approximately = 1%).

3.4 Wastewater degradation by microalgae and final treated water quality

Microalgae have the capacity to consume inorganic and organic nutrients for cell generation. Fig. 3A shows the variation of all carbon species concentration with time in the treated OMW without microalgae (culture medium). It can be observed a TOC slight decrease during the first 200 h of the culture, followed by an increment of TOC and TC in the last stages of the culture, which can be explained by the cell death and ruptures, leading to an increase in the content of organic compounds in the medium. In all experiments, IC concentrations were also decreased with time. As it happened with TOC, the highest reduction levels of the IC concentration occurred during the first 200 h of the culture, which can be explain by the ability of *S. obliquus* to grow mixotrophically assimilating organic compounds as carbon sources while using inorganic compounds as electron donors [17].

Fig. 3B shows the variation of total nitrogen species in the culture media along the culture. It can be observed a decline in the total nitrogen during the first stages, corresponding the most pronounced decrease with the exponential growth of *S. obliquus.* This showed that nitrogen consumption was associated with microalgal growth and its conversion into biomass structures (proteins structure formation). Once the exponential and lineal growth were finished, the concentration of TN in the culture medium remained constant, which can be explained by the cessation of nitrogen assimilation when TN concentration in the culture media was below 2.5 mg/L, corresponding this cessation with the beginning of the stationary phase of growth. Proteins are essential for

microalgae growth. Nutrient deficiency, such as nitrogen starvation, could inhibit protein synthesis and microalgae growth subsequently.

In the experiments, the difference between the total nitrogen concentrations at the beginning and at the end of the culture corresponded to the nitrogen assimilated by *S. obliquus*. This nitrogen removal ranged from 76.3% (5% OMW (v/v)) to 86.5% (100% UW(v/v)).

It can also be seen a reduction in the nitrate-nitrite concentration along the culture, which is completely consumed after 169 hours of culture, which means that all NN present in the culture medium is assimilated by *S. obliquus.*

This results proved the contaminants-removal capacity of microalgae, which are able to assimilate nitrogen from different sources such as nitrate, nitrite, urea or ammonium. This has the mutual advantage of diminishing the harmful effects of wastewaters as well as the reduction of eutrophication in aquatic environments, caused mainly by nitrogen, phosphorus and carbon [18]. Several authors have proved this capacity, such as Wang et al, [15] who reported a decrease in nitrogen of 83% as NH_4^+ by several microalgae species.

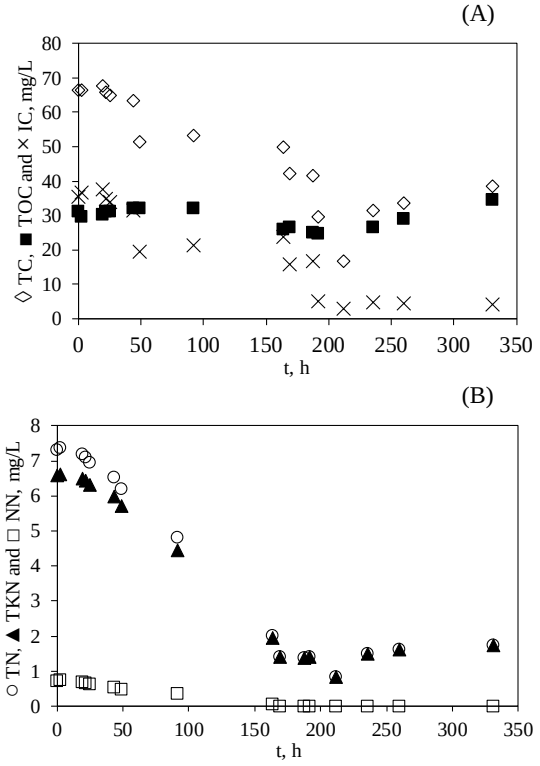

Figure 3: Variation of total carbon species (A) and total nitrogen (B) on the treated wastewater (without algal biomass) during the culture.

Table 3 shows the treated water characteristics after microalgae growth in OMW mixtures. In general, the studied parameters were decreased throughout *S. obliquus* culture, with some exceptions such as turbidity, DQO or TOC in the 5% OMW (v/v) culture in which the presence of cell debris in the final treated water as well as cell ruptures caused an increase of these parameters after *S. obliquus* culture. Nevertheless, cell ruptures occurred to a lesser extent in the 10% OMW (v/v) culture, in which high removal percentages of TOC (60.4%), TC (40.6%) and sulphate (23.2%) were achieved.

With respect to 100% UW characterization it can be observed that primary treatment (microfiltration) allowed higher removal percentages of most parameters in comparison with the secondary treatment (*S. obliquus* culture) which can be explained by the presence of organic matter in the culture media at the end of the culture as a consequence of cell ruptures during the last stages of *S. obliquus* growth. The highest removal percentages during the primary treatment were achieved for phenolic compounds (96.1%), iron (95.6%), orto-phosphate (65.5%), TOC (59.2%), sodium (55.4%) and ammonia (50%). On the other hand, the compounds which were more efficiently removed during the secondary treatment were NN (99.8%), TKN (78.7%), IC (81.6%), sodium (40%) and sulphate (36.9%).

Table III: Characterization of the treated waters obtained after *S. obliquus* cultures. The treated water was separated by centrifugation and microfiltration by membrane with pore size = 0.2 μm).

Parameter	Final treated water (%OMW in UW)		
	0	5	10
EC, mS/cm	1.75	4.46	1.41
Turbidity, FTU	49.7	14.4	0.57
COD, mgO2/L	85.1	691.8	230.6
TPCs, mg/L	4.86	1.10	3.82
TOC, mg/L	12.7	171.3	59.1
TC, mg/L	23.6	176.1	94.8
IC, mg/L	10.9	4.75	35.7
TN, mg/L	3.03	7.39	7.61
NN, mg/L*	0.01	10.2	9.49
NH_4^+, mg/L	0.34	0.76	2.97
Cl^-, mg/L	245.3	2306.3	292.4
SO_4^{-2}, mg/L	420.5	2183.7	667.3
PO_4^{3-}, mg/L	18.8	0.70	2.35
K^+, mg/L	1.04	18.4	19.4
Na^+, mg/L	27.0		0.61
Ca^{2+}, mg/L	1.90	0.13	0.39
Fe, mg/L	0.53	0.28	3.09

**NN is the sum of NO_3+NO_2.*

4 CONCLUSIONS

S. obliquus is able to assimilate nutrients present from wastewaters. This makes possible the use of wastewaters as culture media with the mutual advantage of wastewater treatment and the production of high added value compounds by the microalgae. Urban wastewater and olive oil mill wastewater have a complex composition which hampers its treatment as well as the microalgal biomass growth, since microalgae require a proper nutrient composition in the culture media with carbon, nitrogen and phosphorous sources as the most essential components for biomass generation. In this sense, the mixture of OMW and UW allowed the development of a complete culture medium with all the nutrients required for microalgae growth. Nevertheless, the low protein yields and high carbohydrates content of the final biomass confirmed a nutritional stress situation associated with nitrogen limitation.

The final biomass obtained in the OMW and UW mixtures was characterized by high values of carbohydrate and lipid contents, which could lead to the production of biofuels.

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Research Article

Determination of the Thermal Oxidation Stability and the Kinetic Parameters of Commercial Extra Virgin Olive Oils from Different Varieties

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The use of olive oil with cooking purposes, as final seasoning or within cooked foods is increasing worldwide due to its numerous nutritional and health benefits. These attributes are mainly determined by olive oil chemical composition, which can be altered after thermal processing, oxidation processes, or incorrect practices. For this reason, and due to the numerous factors which have influence in olive oil quality, the correct chemical characterization is highly relevant. In this study, fatty acid composition of four extra virgin olive oil (EVOO) varieties was studied. The major fatty acid (FA) determined was oleic acid (77.1% on average), followed by palmitic (11.5% on average). In addition, thermal oxidation behaviour of the four EVOO samples was studied as an indicator of their quality and stability during thermal processing. This was performed through differential scanning calorimetry (DSC) from a temperature of 40°C at six different heating rates in the range of 0.5–10°C min^{-1}. DSC records showed the same pattern and a small shoulder in the thermo-oxidation peak was present for all samples and all heating rates. The presence of initial and final oxidation products (by monitoring K232 and K270 values, respectively) was discarded according to the International Olive Council method.

1. Introduction

Nowadays, 85% of the total fats consumed in the Mediterranean diet comes from olive oil, a vegetable oil whose consumption is associated with several health benefits such as lower incidence of cardiovascular diseases, cancer, and increased longevity [1]. Most attributes of olive oil quality are determined by its chemical composition as well as the biochemical status of the olive fruit. For the production of high-quality oil, the olives must be harvested without breaking the skins, and they must be processed within 12–24 hours of harvest [2]. Extraction must be made from healthy fruits, avoiding manipulation or treatments which could alter the chemical composition of olive oil during the extraction and storage process [3]. In addition to olive picking, storage, and processing, olive oil composition is

determined by olive tree cultivation, climate, geographical area, etc. [2]. This makes every batch unique and difficult to standardize experimental conditions [4].

The group of major compounds in olive oil composition is triglycerides, which constitute between 92 and 98%. It also contains fatty acids, which contribute 94–96% of the total weight of triglycerides. In this fraction, six are major compounds: oleic (55.2–86.6%), palmitic (6.30–20.9%), linoleic (2.7–20.2%), stearic (0.32–5.33%), palmitoleic (0.32–3.52%), and linolenic (0.11–1.52%). Olive oil is also composed by minor components, fraction constituted by compounds, which derive from triglycerides and liposoluble compounds. This minority fraction can be grouped in the following: diacylglycerols (DAGs), monoacylglycerols (MAGs), free fatty acids (FFAs), oxygenated fatty acids (OFAs), cyclic fatty acids, nonlinear FAs (branched FAs), dimeric FAs, and another compounds such phenols and pigments. The total of these compounds represents between 2 and 5% of the total composition [1].

Olive oil is commonly used as final seasoning, but it is also used with cooking purposes at high temperatures. In this sense, after thermal processing, changes and degradation processes are expected in olive oil; the most usual changes consist of triglyceride polymerization and hydrolysis, fatty acid and sterol oxidation, and Maillard reactions [4]. Oxidation can also alter the flavour and nutritional quality of olive oil due to the loss of beneficial substances and the generation of new toxic compounds including oxidized fatty acids, sterols or TAG polymers, which can have a possible impact on human health and make olive oil less acceptable or unacceptable to consumers [5]. In this sense, differential scanning calorimetry (DSC) is a technique based on the measurement of the energy changes that take place when a sample is heated, cooled, or held isothermally, as well as the determination of the temperature at which these changes occur. These measurements enable the characterization of samples for several complex events such as melting processes or glass transitions [6]. Although DSC has not been established by the International Olive Council as an official method for the determination quality, variety, and geographical origin of olive oil, it has been suggested as a possible method with the advantages of being a fast and easy technique without the necessity of sample pretreatment or use of solvents [7, 8]. According to the official definition, extra virgin olive oil must be extracted by cold and mechanic conditions in an oxygen-free atmosphere in order to preserve the naturally present antioxidants. In refined olive oil, antioxidants are degraded due to refining processes and high temperatures during the olive oil production; as a consequence, the induction period is shorter in lower quality olive oils and can be used to study and compare the thermo-oxidative stability of samples [9]. In this sense, the oxidation of edible oils exhibits the induction period, and at the end of the induction period, the quality of the oil suddenly deteriorates so that the induction period is considered as a measurement of the oil stability [10].

In addition to DSC, spectroscopic techniques are suitable for quality control of olive oil. Fluorescence spectroscopy is a simple, rapid, economic, and nondestructive technique which is applied to determine the stage of decomposition of oils [11]. The K232 and K270 values are spectrophotometric measures for quantifying the UV absorption at 232 nm and 270 nm, respectively. It provides information about the quality of the fat, the conservation status of the oil, and any deterioration occurred during the technological processes [2]. It corresponds to the maximum absorption of the conjugated dienes and trienes, and it is expressed as specific extinctions coefficients [12].

Other technique that can be found in the literature is "Rancimat stability" which consists of exposing the olive oil to forced oxidation at 100°C until its maximum oxidation, measuring the time required for an abrupt change in conductivity from an aqueous solution where the volatile compounds carried by the oil were collected. The duration time of this period is considered as the index of resistance to rancidness of the fat being assayed [13].

In this work, the quality and stability of different varieties of olive oil were studied. The fatty acid profiles of four commercial EVOO were determined. The thermal oxidation stability and the kinetic parameters related to the oxidation process by DSC were evaluated. The specific UV extinction coefficients (K232 and K270) were determined to study the presence of oxidation products.

2. Materials and Methods

2.1. Samples. Four extra virgin olive oils samples of different brands were bought in a local store in Spain (Table 1). The samples were kept in a refrigerator at 4°C until the time of analysis.

2.2. Fatty Acid Profiles Determination. A mass between 0.10 and 0.30 g of each sample was weighted and dissolved in heptane in a reaction vessel with volume capacity equal to 1 cm^3. After the sample dilution, 100 μl of sodium methoxide, the transesterification agent, was added. The time of the transesterification reaction had a duration between 15 and 20 minutes. Then, an excess of methanolic HCl (typically 100 μl) was added and the reaction was carried out at room temperature for 45 minutes. The upper heptane layer was separated and injected into the gas chromatograph [14].

Fatty acid composition was determined by the gas chromatograph GC-7890 (Agilent, USA) with a FID detector and capillary column DB-23 (60 m × 0.25 mm, with 0.25 μm stationary phase of poly(cyanopropylmethyl siloxane)). A volume of 1 μL of FAME and heptane was injected. Carrier gas flow rate was equal to 16.4 $cm^3\,min^{-1}$ and pressure = 220 kPa. Programming chromatographic temperature was set at the initial value of 150°C (held for 6 min), followed by a heating rate of 5°C min^{-1} up to 170°C and heating rate of 6°C min^{-1} up to 220°C (held for 6 min). Next stage was a heating rate of 6°C min^{-1} at 220°C for 1 min and finally, heating rate of 30°C min^{-1} up to 240°C for 10 minutes. FID hydrogen flow and airflow rate were 40 $cm^3\,min^{-1}$ and 450 $cm^3\,min^{-1}$, respectively.

2.3. Differential Scanning Calorimetry. The DSC analysis was conducted on a differential scanning calorimeter, Shimadzu

Table 1: Identification of extra virgin olive oil samples analyzed.

Variety	ID	Origin
Coupage Changlot Real and Arbosana	C + A	Spain
Manzanilla Cacereña	Ma	Spain
Koroneiki	Ko	Greece*
Arbequina	Ar	Spain

All samples have been produced using the two-phase extraction system. *Olives grown in Spain.

DSC-60 (Tokyo, Japan) equipped with an automatic gas switching unit. The temperature scale of the instrument was calibrated to the melting points of enzyl, In, Sn, and Pb. The measurement of thermo-oxidative stability was carried out in nonisothermal mode with linear heating. Samples of 3.5–4.5 mg were placed into open aluminium pans and heated in dynamic air atmosphere (50 mL min^{-1}) from 40°C at 6 different heating rates in the range of 0.5–10°C min^{-1}. Each measurement was terminated once an exothermic peak corresponding to thermal oxidation was observed.

2.4. Determination of Specific UV Extinction Coefficients (K232 and K270). The measurement was performed through UV/VIS spectrophotometry with a UV-1600 series spectrophotometer (VWR, Leuven, Belgium). Absorbance within a 200 to 800 nm spectral range was measured at 1 nm spectral resolution using a 1 cm path length quartz cell, in the region of 200–380 nm.

Olive oil samples were perfectly homogeneous without any suspended impurities. A mass of 0.25–0.30 g was weighted and diluted to a one percent solution in cyclohexane. Spectrophotometric analysis of olive oil was performed in accordance with the official method in the Commission Regulation (EC) [15], which involves the determination of the specific extinction in cyclohexane at wavelength of 232 and 270 nm and the determination of K232 and K270 according to the following equation:

$$K_\lambda = \frac{A_\lambda}{c \cdot L}, \quad (1)$$

where K_λ is the extinction coefficient, A_λ is the absorbance, c is the concentration of the sample in the solvent in g/100 mL, and L is the path length of the cuvette in cm.

3. Results and Discussion

3.1. Fatty Acids Composition of Extra Virgin Olive Oils. The fatty acid (FA) profile of olive oil is highly relevant, and it is considered as a parameter to characterize the diverse olive varieties since the quality of the fat has a direct impact on oil quality and thus, on consumer health [16]. In addition to the clinical relevance and the nutritional value of some FA such as oleic acid, FAs are also responsible for the presence of desired and undesired volatile compounds, which have a direct influence on the positive or negative sensory perceptions in olive oil. Lipoxygenase (LOX) pathways generate most of the desired volatile aroma compounds (C5 and C6 compounds and saturated aldehydes). A series of oxidative reactions result in a large variety of metabolites from polyunsaturated FA, linoleic and linolenic acids being the main initial substrates. The importance of the FA profile is, therefore, due to the fact that high and poor quality olive oils differ by their content in these compounds derived from FA [17].

Fatty acid content of olive oils is highly variable since it is affected by numerous factors such as production and cultivation area, latitude, climate, fruit ripeness, genetic factors, etc. Environmental factors are the ones that have a greater influence on FA composition of olive oils, temperature being the one that plays an essential role in the FA profile of olive oil, since temperature regulates fatty acid desaturases. Polyunsaturated fatty acids are present in greater proportions at low temperatures [18]. In this sense, differences in the FA profile of the four studied EVOO can be explained by the different geographical areas and climate conditions in which olive fruits were grown. In addition, several agronomic, processing, and environmental variables such as degree of ripeness or storage and processing conditions have a direct influence on the olive oil chemical composition [19].

Table 2 shows the fatty acid profile (%, weight) of the different EVOO. Determined fatty acids have been grouped as total saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids. The major fatty acid percentage found was oleic acid (C18 : 1) as expected. This fatty acid content ranged from 75.2% (Ar) to 79.9% (Ko), followed by palmitic acid (C16 : 0) which ranged from 10.4% (Ko) to 12.9% (Ar), linoleic acid (C18 : 2), from 5.09% (Ko) to 8.27% (Ar), stearic acid (C18 : 0), which ranged from 1.85% in Ar to 2.08% in C + A, and linolenic acid (C18 : 3) whose content ranged from 0.59% in Ar to 2.82% in C + A. Other fatty acids such as palmitoleic acid (C16 : 1, 0.86% on average), gadoleic acid (C20 : 1; 1.24% on average), behenic acid (C22 : 0; 0.50% on average), and arachidic acid (C20 : 0; 0.27% on average) were detected in all EVOO samples and found at a concentration of less than 1%. In general, no significant variation was detected in the fatty acids composition of the different EVOO studied, showed by the standard deviation values, which varied from 0.10 (C20 : 0) to 2.23 (C18 : 1).

Saturated fatty acids comprised about 13.6% of the total fatty acids, whereas monounsaturated and polyunsaturated fatty acids represented 77.4% and 8.98%, respectively. Total unsaturated fatty acids (MUFA + PUFA) in olive oil constituted 86.4% of the total. These fractions corresponded, almost entirely, to oleic acid, while palmitic acid represented the greatest proportion of SFA.

Regarding FA composition, significant differences exist between olive oil and other vegetable oils. In this sense, Li et al. [20] determined the fatty acid profile of palm oil, rapeseed oil, sunflower oil, and linseed oil. Compared to these four vegetables oils, it must be highlighted the higher oleic acid content in the four EVOO studied in this work (77.1% in average) in comparison with rapeseed, palm, sunflower, and linseed oil, whose content in oleic acid was notably lower: 46.3%, 33.6%, 13.6%, and 1.2%, respectively. In addition, palmitic acid, the second most abundant FA in olive oil (11.5% on average), was found in notably lower percentages in sunflower oil (3.89%), linseed oil (3.12%), and

Table 2: Fatty acids profile determined in four commercial samples of EVOO.

Fatty acids	EVOO sample				Average	SD
	C + A	Ma	Ko	Ar		
C16 : 0 (palmitic)	11.2	11.6	10.4	12.9	11.5	1.03
C16 : 1 (palmitoleic)	0.80	0.88	0.67	1.08	0.86	0.17
C18 : 0 (stearic)	2.08	1.97	2.05	1.85	1.99	0.11
C18 : 1 (oleic)	75.4	77.7	79.9	75.2	77.1	2.23
C18 : 2 (linoleic)	6.16	6.26	5.09	8.27	6.44	1.33
C20 : 0 (arachidic)	0.33	0.36	0.28	0.13	0.27	0.10
C20 : 1 (gadoleic)	1.24	n.d	n.d	n.d	1.24	
C18 : 3 (linolenic)	2.82	0.84	0.89	0.59	1.29	1.03
C22 : 0 (behenic)	n.d	0.36	0.65	n.d	0.50	0.20
$\sum$ SFA*	13.6	14.3	13.4	14.9	14.1	0.67
$\sum$ MUFA**	77.4	78.6	80.6	76.3	78.2	1.84
$\sum$ PUFA***	8.98	7.10	5.98	8.85	7.73	1.45

*Sum of saturated fatty acids. **Sum of monounsaturated fatty acids. ***Sum of polyunsaturated fatty acids.

rapeseed oil (2.69%), nevertheless, higher content of this FA was found in palm oil (29.3%) in comparison with EVOO. Content of linoleic and stearic acids in EVOO (6.44% and 1.99% on average, respectively) were lower in comparison with the other vegetable oils, whose content ranged from 8.12% (palm oil) to 51.9% (sunflower oil) for linoleic acid and between 1.51% (rapeseed oil) and 3.59% (palm oil) for stearic acid. Linolenic acid was only found in rapeseed and linseed oil, at a concentration of less than 1%. Myristic acid (C14 : 0), which was not found in olive oil, was found at 0.43% in palm oil.

Similarly, Berasategi et al. [21] studied avocado oil fatty acid composition. This oil consumption and production is significantly growing in recent years due to its beneficial health properties attributed to its high concentration of oleic acid, antioxidant vitamins, and phytosterols. This study showed that MUFA content in avocado oil was equal to 68.4% with a total content of 54.4% of oleic acid of total FA. These values are much lower in comparison with the EVOO studied in this work, which contained 78.2% on average of MUFA and oleic acid ranging from 75.2% to 79.9%. On the contrary, palmitoleic acid, whose average content in EVOO was equal to 0.86%, was found at higher concentration (7.88%) in avocado oil. The importance of MUFA content can be explained by its relation with higher concentration of minor compounds with antioxidant and hypocholesterolemic effects [21].

On the other hand, higher PUFA content was found in avocado oil (11.8%) in comparison with EVOO (7.73%). Within this group, EVOO contained 2-fold the amount of linolenic acid present in avocado oil (0.61%). Lastly, SFA content in avocado was equal to 11.8% in comparison with 7.73% in EVOO and with the main differences in palmitic and stearic acids, whose contents were equal to 18.7 and 0.51%, respectively.

3.2. Differential Scanning Calorimetry. The standard tests used for the determination of the induction period are predominantly carried out under isothermal conditions, i.e., the oxidation induction time is measured. However, under isothermal conditions, the oxidation peak measured is often flat and its onset, corresponding to the end of induction period, cannot be determined unambiguously. On the contrary, in the experiments with constant heating rate, the oxidation peak is distinct and the onset oxidation temperature can be measured accurately and unambiguously. In our previous work, a theory of the kinetic description of induction periods from nonisothermal measurements has been outlined [22] and applied for the study of thermo-oxidation of edible oils [10]. For the treatment of experimental DSC data, it was applied the procedure from the latter citation.

The DSC records of nonisothermal thermo-oxidation of olive oil C + A are depicted in Figure 1; the other EVOOs studied exhibited similar pattern. The peak corresponding to thermo-oxidation exhibits a small shoulder near its onset. The shoulder is present for all samples and for all heating rates employed; therefore, the values of oxidation onset temperatures, T_i, were evaluated as its onset extrapolated to the baseline. It can be seen from Figure 1 that higher heating rate always leads to higher oxidation onset temperature. Šimon [22] demonstrated that employing a non-Arrhenian dependence of the reaction rate on temperature, $k\ (T) = A' \exp(DT)$, and assuming the same conversion for all heating rates, the dependence of oxidation onset temperature (T_i) on the heating rate can be described by the following equation:

$$T_i = \frac{1}{D} \ln (AD\beta + 1), \tag{2}$$

where "β" is the heating rate in °C min^{-1} and "A" and "D" are kinetic parameters of thermo-oxidation. Once the values of the kinetic parameters are determined from a series of experiments carried out at different heating rates, the oxidation induction time (OIT) can be calculated as

$$\mathrm{OIT\,(T)} = A \exp(-\mathrm{DT}). \tag{3}$$

The evaluated oxidation onset temperatures for each oil at various heating rates are listed in Table 3. These T_i vs. β dependences were further analyzed to estimate the kinetic parameters employing nonlinear least squares method applied to equation (3); the resulting parameters are listed in Table 4. Figure 2 depicts a typical result of the least squares fitting procedure.

The kinetic parameters obtained from the treatment of nonisothermal data were used to predict the values of OIT. The prediction of the values of oxidation induction time, OITs, based on equation (3) for each olive oil are presented in Figure 3. Two representative temperatures were chosen (25°C and 150°C). The lower temperature represents the usual storage conditions. However, care should be taken since both representative temperatures chosen (25°C and 150°C) are outside the experimental range of DSC measurements. The higher representative temperature chosen (150°C) is much closer to the experimentally investigated temperature range and the corresponding OIT values are expected to be both more precise and accurate.

Figure 3 shows that all the OITs values predicted at 150°C lie in a relatively narrow range of 30 to 50 min with oil Arbequina being least stable. Considering the OITs

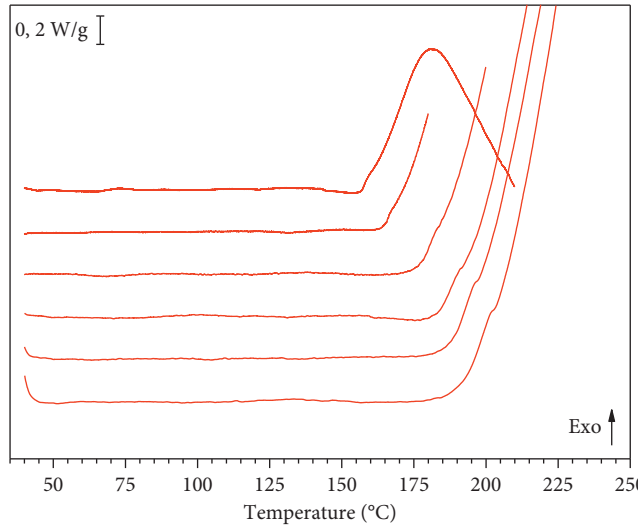

Figure 1: Nonisothermal DSC records of thermal oxidation (olive oil: C + A) obtained for different heating rates (from top to bottom: 0.5, 1, 3, 5, 7, and 10°C/min).

Table 3: Oxidation onset temperatures of olive oils for various heating rates.

β (°C min^{-1})	T_i (°C)			
	C + A	Ma	Ko	Ar
0.5	156.7	156.2	158.0	152.3
1	164.6	165.3	167.8	161.3
3	176.7	176.0	180.5	174.9
5	182.8	181.6	189.0	182.6
7	187.3	187.9	193.3	186.0
10	191.1	192.9	196.9	190.0

Table 4: Values of the kinetic parameters with their standard errors.

	C + A	Ma	Ko	Ar
ln A/min	40.51 ± 0.43	39.47 ± 1.09	36.23 ± 0.80	36.70 ± 0.56
D (K^{-1})	0.08697 ± 0.00099	0.0846 ± 0.0024	0.0764 ± 0.0018	0.0786 ± 0.0013

uncertainty, all the olive oils exhibit approximately the same high-temperature thermo-oxidative stability.

Results for 25°C also suggest that Arbequina is the least stable oil, and the Coupage Changlot Real and Arbosana has about four times longer shelf life—the differences between the oils are now much more pronounced. However, it should be kept in mind that the temperature (25°C) lies far away from the experimental range, and nonlinear extrapolation affects both accuracy and precision of the results (as demonstrated by much longer error bars compared to high-temperature prediction).

Similarly, Li et al. [20] studied thermal oxidation stability of four different vegetable oils (palm, rapeseed, sunflower, and linseed oil) through DCS at different heating rates (1, 5, 7.5, 10, 15, and 20°C/min). According to T_i obtained for the different oils, the following order for oxidation stability was obtained: palm oil > rapeseed oil > sunflower oil > linseed oil. When comparing Li et al.'s [20] results with the present study, it can be concluded that for all heating rates, the four vegetable oils showed higher T_i in comparison with the EVOO studied in the present work. T_i at a heating rate of 10°C/min was equal to 250.2, 233.3, 221.1, and 202.9°C for palm, rapeseed, sunflower, and linseed oil, respectively. In contrast, T_i values between 190 and 196.9°C were obtained for the EVOO samples at the same conditions. Similar pattern was observed for all heating rates. In addition, similar behaviour was registered in both studies when comparing thermal decomposition profiles at different heating rates: higher heating rate resulted in higher degradation rate and increased T_i.

Differences in oxidation stability of these vegetable oils are directly related to FA composition: vegetable oils with higher UFA content are usually less stable than those with higher SFA proportion. This can be explained by FA chemical structure, determined by chain length, unsaturation degree, and ramifications. Oxidation mostly occurs in double bonds; for this reason, FAs with higher unsaturation degree are more prone to oxidation and less stable, as a consequence, than SFA [23, 24].

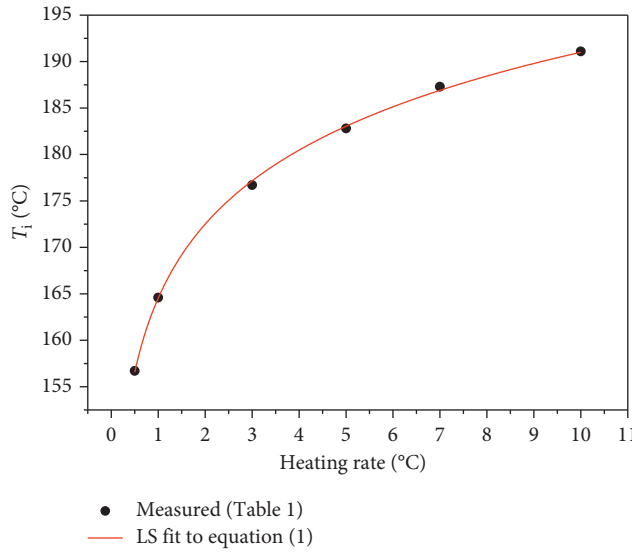

Figure 2: Experimental and fitted dependences of the oxidation onset temperatures on the heating rate (olive oil: C + A).

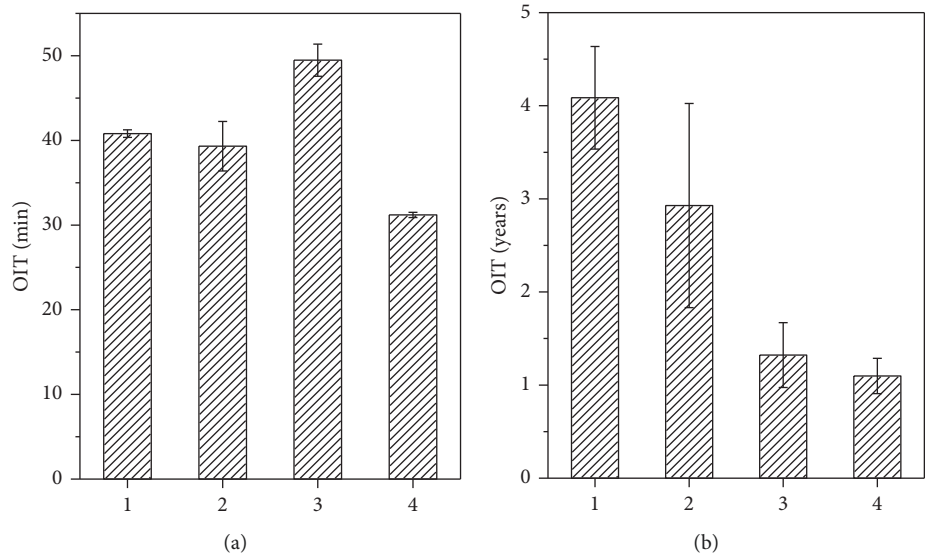

Figure 3: OITs for olive oils: (1) C + A, (2) Ma, (3) Ko, and (4) Ar predicted from nonisothermal experiments using equation (3). (a) 150°C. (b) 50°C.

3.3. Ultraviolet Spectrophotometry. The four EVOO varieties studied showed similar UV spectra in the UV and visible range (Figure 4). Evaluation of the spectra of the four samples according to equation (1) yields the values summarized in Table 5. As shown, all olive oils fulfill the criteria for extra virgin olive oil laid down by the International Olive Oil Council and the Commission Regulation [15] since K232 and K270 values were lower than the limits established (2.50 and 0.22, respectively).

K232 is related to the presence of hydroperoxides, conjugated dienes, carboxylic compounds, and conjugated trienes. On the other hand, K270 is dependent on the secondary products formed from the oxidation products detected at 232 nm [11, 26]. Therefore, results indicated the absence of oxidation products in the olive oils studied as well as the absence of refining oil in the commercial EVOO samples.

Allouche et al. [27] studied the evolution of K232 and K270 values of two extra virgin olive oils from Arbequina and Picual cultivars during heating at 180°C. Results showed that both indexes increased notably during the heating process, obtaining the higher values for Arbequina oil. Similarly, it was experimentally proved in [11] that during oil oxidation, high levels of peroxides are generated from primary oxidation compounds, resulting in higher K232 and K270 values and fluorescence spectra with peaks in the 415–600 nm region. In addition, it was demonstrated in this study that the combination of fluorescence techniques with multivariate analysis is a suitable method to characterize

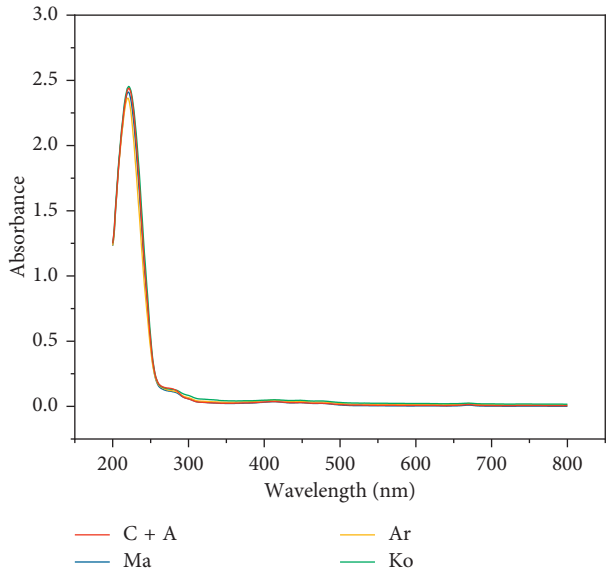

Figure 4: UV spectra for the four olive oil varieties studied.

Table 5: K_{232} and K_{270} values of the analyzed EVOO samples.

	K_{232}	K_{270}
Extra virgin olive oil criteria*	≤2.50	≤0.20
Changlot Real + Arbosana	1.95	0.14
Manzanilla Cacereña	1.88	0.12
Koroneiki	1.71	0.13
Arbequina	2.02	0.14

*Maximum values allowed according the Commission Regulation (CEE) no. 2568/91: K232 ≤ 2.50 and K270 ≤ 0.20 [25].

olive oil on the basis of the main quality parameters of olive oil: peroxide value, K232, K270, and acidity.

The suitability of K232 and K270 to determine the quality and conservation status of vegetable oils was also proved by Rodrigues et al. [28]. In this work, oil from *Jatropha curcas* L seeds was stored for 42 days, at 35°C and 75% or 92% relative humidity (RH). Results showed that higher RH resulted in a higher increment in K232 and K270 values. Regarding K232, an increase of 0.029 absorbance units/day was observed at 75% RH; nevertheless, a faster increase was observed at 92% RH (0.059 absorbance units/day). Similar results were obtained for K270, showing an increase from 0.07 to 0.22 after storage in higher humidity conditions.

4. Conclusions

Authentication and traceability of extra virgin olive oils are highly in demand in the market. The International Olive Oil Council and the Commission Regulation [15] has defined the quality of olive oil according to a series of parameters such as free fatty acids content and UV-specific extinction coefficients (K232 and K270). These parameters were determined in this work; results showed that oleic acid is the most abundant in the four EVOO (77.1% on average), followed by palmitic (11.5% on average). The importance of FA profile is due to its high contribution to olive oil oxidative stability. K232 and K270 values confirmed the absence of oxidation primary and secondary products.

In addition, the results showed that oil analysis can be performed with differential scanning calorimetry, an alternative technique for the evaluation of olive oil quality and stability as well as the determination of the heating effect on olive oil. DSC is an efficient, fast, accurate, and environmentally friendly method for the identification of peaks related to olive oil chemical composition. Nevertheless, in terms of authenticity, the information provided by the DSC analysis is not enough to detect adulterated olive oils due to the large number of possible adulterants [1].

In the four different EVOO varieties studied, DSC provided thermal fingerprints of the samples. For all heating rates, the peak corresponding to thermo-oxidation exhibits a small shoulder near its onset and all samples shown similar DSC record. It also can be concluded from the analysis of the T_i vs. β dependences that, for all samples, higher heating rate always leads to higher oxidation onset temperature. When comparing results obtained at two representative temperatures (25°C and 150°C), higher temperature is much closer to the experimentally investigated temperature range, as a consequence, OIT values obtained are more precise and accurate, exhibiting all the oils approximately the same thermo-oxidative stability. Much longer error bars as a consequence of less accuracy and precision of the results are obtained at 25°C.

It can, therefore, be concluded that the control of storage conditions of olive oil (temperature, humidity, etc.) is extremely relevant in order to preserve its quality. Evaluation of FA profile, K232 and K270 values, and T_i through DSC is a suitable, simple, and accurate technique to predict the quality, conservation status, and oxidation stability of different vegetable oils.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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